DOCTORAL THESIS

Utilization of Extreme Drug Resistance Testing in Malignant Melanoma: new is not Always Better.

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UTLIZATION OF EXTREME DRUG RESISTANCE TESTING IN MALIGNANT MELANOMA: NEW IS NOT ALWAYS BETTER

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This thesis represents my own work and contains no material which has been previously submitted for a degree or diploma at this University or any other institution, except where acknowledgement is made

*Signature*_____ *Date*_____

To Alice and all my heroes

In appreciation to my parents, grandparents, siblings and nieces, my friends and to Joe

Gracious a mi amor verdad

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Abstract

This research considers the treatment of malignant melanoma. Data were collected from patient records for 78 individuals treated within the Yale Cancer Center Melanoma Unit. The patients were diagnosed with malignant melanoma prior to 1994 and progressed to stage III or IV disease before their deaths.

Due to the rapid progression of malignant melanoma, treatments are initiated at the time of diagnosis. Results of experimental Extreme Drug Resistance (EDR) tests subsequently become available. Physicians are warned the test results are not intended to guide therapy; however, assay directed therapies arguably result in better outcomes with other cancers. Thus, the question arises of whether the use of these tests might benefit patients in this context.

This study evaluates the treatment decisions made using a multi-disciplinary approach within the Yale Cancer Center Melanoma Unit regarding patients with malignant melanoma relative to information contained in EDR tests conducted by Oncotech Inc. Within this comparison, three specific outcomes consistent with hypotheses of the study were assessed: the utilization of test results, drug toxicity and cost effectiveness and survival.

Results were found to suggest that the initial treatment decisions of the Yale Cancer Center Melanoma Unit were in accord with tests results that were received henceforth for 74 of 78 patients. Two of those patients were in terminal stages of the disease thus treatments were unchanged; however two patients received a change in therapy.

It is suggested that physicians made use of the tests as they became available. However, only two patients with therapies altered by the test results were shown to face reduced costs, drug toxicity, or have the benefit of improved survival. From the patient data collected, four patients receiving drugs to which their tumors exhibited EDR were found to exhibit shorter survival times. Literature review studies conducted to evaluate physician treatment approach and patient preference rate favorably the consideration of quality of life issues. The principle finding of this observational study which focuses upon the development of the Yale Cancer Center Melanoma Unit, suggest that a multi-disciplinary approach to the treatment of malignant melanoma may offer quality of life benefits to the patient.

INTRODUCTION

Melanoma is the most common malignancy in humans (Gallagher, 2003). Melanoma is the leading cause of death from skin tumors worldwide with an annual increase in incidence over the past decade (Stahl et al., 2004). The incidence of malignant melanoma in the United States has doubled each decade for the past fifty years (Berger et al., 2004; Tarhini and Agarwala, 2005) and the number of people that have developed melanoma is increasing at a faster rate than any other form of cancer (Kuhn and Harke, 2002). In 2005, melanoma is estimated to affect 55, 000 American lives. Of these, 7,700 are estimated to die from the disease (Tarhini and Agarwala, 2005). In the United States, the incidence of melanoma in women is increasing at a rate second only to lung cancer. Early stage melanoma is curable however advanced metastatic melanoma is almost uniformly fatal (Agarwala, 2003). Approximately 20% of people who develop the cutaneous form of melanoma will progress to malignant metastatic disease (Buzaid and Adkins, 2001). Patients with stage IV melanoma have an overall five year survival of less than 10% and a median survival of 8.5 months (Prignano et al., 2002).

Immunological approaches have yielded the only newly approved agents for malignant melanoma in 30 years which includes high-dose bolus Interluekin-2 with limited durable clinical response rates (Tarhini and Agarwala, 2005). To date, no standard adjuvant therapy has demonstrated increased overall survival (Ascierto et al., 2005). The agents clinically available for malignant melanoma are associated with high cost, drug toxicity and drug resistance. Despite a number of novel therapeutics undergoing active clinical investigation (Agarwal, 2003), malignant melanoma is at

present intractable. The single greatest deterrent to effective treatment is the drug resistance which malignant melanoma exerts.

The research contained in this document focuses on the utilization of diagnostic testing for drug resistance in malignant melanoma. The primary focus of the research is the utilization of in vitro extreme drug resistance (EDR) micro-arrays by the physicians who treat malignant melanoma. The micro-array tests are performed in clinical laboratories to determine drug resistance on individual tumor tissue samples. In vitro EDR testing is commercially available to physicians who treat malignancies to guide treatment choices for the individual patient. Parameters including accuracy, feasibility and effectiveness of the micro-array EDR testing method are the subject of the research. While the research in the document is focused principally on utilization of the EDR in vitro micro-array testing, it also contains research for in vitro testing of biomarkers in malignant melanoma.

The research contained in the document was conducted at the Yale Melanoma Cancer Center. The center is comprised of physicians who treat stage III and IV malignant melanoma. The physicians in the center consider the utilization of in vitro EDR testing in their treatment determination for malignant melanoma. The practice style employed by the physicians encompasses a multi-disciplinary approach which is inclusive to this research. The aim of the research was to evaluate the consideration of the utilization of the in vitro EDR tests and the practice style employed by the physicians at the Yale Cancer Center for a disease which demands a departure from conventional medical practice.

The research deliberates on the decision making process of therapeutic choice for malignant melanoma and the challenges inherent to the variety of novel diagnostic tests available to augment this process. The research includes discussion of the relative predictive accuracy or value on in vitro micro-assays and biomarker testing for malignant melanoma. The document is foreshadowed by the overarching global problem of drug resistance. Disease processes and diagnostic tests that address issues of drug resistance are provided in the document to highlight the emerging global challenges facing clinicians who treat chronic illness and the aspects which parallel malignant melanoma.

The document discusses the problematic issues of medical surveillance in drug resistance research. A conventional problem with medical surveillance programs that apply diagnostic testing and biomarkers is determining the optimal frequency of such testing to minimize adverse health effects and cost (Judd et al., 2003). Considerable economic and health problems emanate from drug resistance as seen in bacterial infection, viral illness and in oncologic disease. Research to accurately quantify problems of drug resistance and proposals to evaluate practicable solutions are needed (Okeke, 2005). This research attempts to identify economic issues regarding in vitro testing and the difficulties and realities of clinician choice in the therapeutics of malignant melanoma. The research conducted as contained in this document suggests the need for further study to identify and quantify the diagnostic testing and therapeutic intervention for malignant melanoma.

Vaccines are being tested in patients with metastatic melanoma to determine their immune effects and to define their activity in combination with other immunotherapeutic agents. The two most widely investigated immunotherapy drugs for melanoma are

interferon-alpha (INF-alpha) and interleukin-2 (IL-2). Their therapeutic success in malignant melanoma is limited. Recombinant IL-2 demonstrates an overall response rate of 15-20% with resultant complete and durable remissions in only six per cent of patients (Agarwala, 2003). Additionally the economic burden and drug toxicity of recombinant IL-2 is excessive and is a concern shared by both the patient and treating physician (Sun and Schucter; 2001; Garle and Ergentler; 2004). This study will highlight physician choice in therapeutic treatment for malignant melanoma.

Melanoma cells exhibit a high level of intrinsic or acquired resistance to the cytotoxic agents often associated with the over-expression of drug transporters (Molinari et al., 2005). Malignant melanoma represents a very difficult challenge for the medical oncologist despite varied chemotherapeutic approaches. Treatment of melanoma in the stage of distant metastasis aims on palliation and achievement of durable tumor remission by prolongation of survival (Crosby et al., 2000; Garle and Ergentler, 2004).

If metastasis is confined to one organ system and is removable, surgery remains the treatment of choice (Garle and Ergentler, 2004). Unfortunately, a majority of post-surgical metastasis relapses and succumbs to distant disease (Spanknebel and Kaufmann, 2004). In limited metastasis, radiation therapy for bone and brain metastasis offers palliation (Garle and Ergentler, 2004). Whole-brain radiotherapy has had limited success to alleviate palliative symptoms (Eedy, 2003). According to the 3rd revision of the Guidelines for Melanoma (van Everdingen et al., 2005) treatment for metastatic melanoma should include inclusion into clinical trials.

Malignant melanoma is aggressive and refractarious with a futile prognosis. In patients who progress to advanced melanoma, only low response rates (between 10-15%)

have been achieved by the single-agent cytostatics, leading to a mean five-year survival in less than five percent of patients (Buzaid and Atkins, 2001; Garle and Ergentler, 2004) with durable remissions occurring in less than two percent of the patients (Buzaid and Atkins, 2001). More aggressive treatment regimens using multi-drug chemotherapy yielded response rates of up to 40%, but failed to show a significant benefit in overall survival compared to single agent therapy (Buzaid and Atkins, 2001; Garle and Ergentler, 2004). Monotherapy with dacarbazine, temozolomide, fotemustine or vindestine or its combination with Interferon-alpha are the current preferred agents; however all have failed to prolong survival (Garbe and Eigentler, 2004). This document further discusses issues of drug treatment in advanced melanoma.

The untold velocity which malignant melanoma progresses in the human body undermines clinical attempts in chemotherapeutic intervention. Given the rapid transit of the tumor's activity, timing and accuracy of treatment are ideal but generally unrealistic parameters in treatment consideration. The empiric approach to chemotherapy has limitations. The malignancy of melanoma is felonious and its tenancy is proven by its extreme drug resistance. Individually the tumor burden is so high as to resist drug effectiveness. It is very difficult to determine on case-by-case basis which drug or drug agents are effective. It is difficult to determine drug effectiveness when factoring in confounding variables of co-morbidity, drug toxicity and rapid progression from diagnosis to death. In effect the human body (in vivo) of a patient with advanced melanoma becomes a less than ideal medium from which to observe drug behavior (sensitivity and resistance) and drug success.

A minimum of two months of in vivo chemotherapeutic administration is needed in each individual patient to determine drug response and high tumor resistance to cytotoxic agents for malignant melanoma and most other malignancies (Fruehauf and Bosanquet, 1993). In malignant melanoma the interval between diagnosis and death does not lend itself to accurate or feasible in vivo testing for individual tumor drug resistance. Several pernicious scenarios can arise alone or in concert during this interval. During this interval many issues are salient in the treatment determination.

Quality of life is one primary concern to the treating physician and to the patient with malignant melanoma. Quality of life encompasses many facets and components and is a consideration that is difficult to measure clinically as it varies considerably from individual to individual yet its importance is pervasive in devising a treatment plan. Quality of life issues constitute personal issues ranging from economic burden, family, employment, religion, and coping skills. The prognosis of the disease and the side effect profile of the treatment drugs confer psychological challenge, morbidity, and often worsening of co-morbidity. Their effects can be toxic and without benefit.

The range of potential side effects from cytotoxic agents and particularly immunotherapy include blood dyscrasias which may lead to mortality. It may become difficult to decipher clinically whether systemic affects are due drug toxicity or disease progression. Disease progression may occur during the evaluation period to first-line therapy. It may be the case that ineffective therapy may induce cross-resistance to subsequent agents that initially may have been effective, thus decreasing the probability of a clinical response. These results can be reversible or non-reversible stemming solely drug administration. The clinical course of illness in the progression of malignant

melanoma induces challenge in clinical treatment determination. This challenge is enhanced by the economic burden which accompanies the consideration of drug choice. The therapeutic choices made by the treating physician are not made in exclusion to the cost to the patient and should not be made in exclusion to the cost of experimental trial to the society.

The economic burden for treatment of malignant melanoma for the patient and society is high. The cost for an individual patient may exceed financial insurance coverage allotment and individual finances may be absent from time of diagnosis. The cost includes hospitalization necessary to administer some types of therapy, the cost of the drugs itself, the cost of toxic effects and cost to quality of life.

Cost as a variable is inclusive to scientific drug discovery with many permutations. It is not a novel concept that the treatment of cancer can be costly for the individual and for society. Yet as the arena of healthcare continues to evolve as a prime concern on global scale, the ante is raised as is people's awareness of limited financial resources. Insurance coverage is not consistent as people would like especially when confronted with a devastating illness such as malignant melanoma. As such, the cost of cancer treatment and the accompanying insurance coverage renders itself less bedfellows. The drug resistance seen in advanced melanoma and the actual cost to attempt to treat it are in direct inverse proportion to the insurance coverage realized for its course.

Precision and economics of drug testing become very important tools as the climate of healthcare continues and as people continue to live longer and demand healthier lifestyles. There is limited research on malignant melanoma and cost of treatment. Lafma and Grob (2003) conducted a literature review of clinical trials and

economic studies published on the use of IFN-α as an adjuvant therapy in stage II-III (AJCC 1992) malignant melanoma. The authors selected large clinical trials with sufficient follow-up to assess efficacy of trial organization. Medico-economic studies, based on the results of several of these trials, were analyzed to estimate the cost-effectiveness ratios of IFN in this disease. Interferon-α as adjuvant therapy used to treat malignant melanoma, demonstrated efficacy with high-dose regimens in patients with overt regional nodal disease (high-risk) and with low-dose regimens in stage IIA and–B patients without clinically detectable nodes (intermediate-risk). Studies such as these can offer economic analysis performed in varied settings and using several methods to extrapolate clinical results which can lend data and in this case are producing similar results of the extra costs for IFN-associated treatment. The incremental cost-effectiveness ratios provided in the study were U.S. $50,000/per life/per year gained in widely used medical strategies for different disease settings. This study suggests the recommendation of IFN-α therapy in malignant melanoma, specifically high doses in high-risk patients and low doses in intermediate-risk patients. In order that the treatment decision in whether or not to treat is made, however, the patient will need to be informed that IFN (and IL-2) may only delay progression of disease with the possibility of any curative effect being uncertain. This limited effect must be balanced with the potential impact on quality of life from the high side-effect profile of IFN and especially IL-2 and with the findings by Lafma and Grob that many patients in whom low doses are indicated would not recur in the absence of treatment.

The cost of drug resistance can be reduced by effective diagnostic tools that improve drug treatment success. Metastatic melanoma exhibits a highly metastatic

character and resistance to radio and chemotherapy (Dai et al., 2004). Diagnostic tools that improve drug response and address drug resistance are needed to augment the diagnosis and treatment of malignant melanoma. The physicians who treat advanced melanoma are charged with the responsibility to consider diagnostic tools that are economic, feasible, assessable, accurate and effective. The treating physician must chose wisely from diagnostic tools that address a time-sensitive treatment plan yet one that focuses on the individual patient. Diagnostic tools that satisfy these variables for clinical utilization in malignant melanoma are limited or nearly non-existent.

This document and the research contained there within examine the use of a diagnostic tool under consideration and availability to the physicians to enhance empiric treatment of the disease. The remainder of the document will describe in vitro EDR testing in malignant melanoma. In short, the assays are used to identify the most effective treatment for an individual when many options exist, much like the use of estrogen-receptor expression and tamoxifen (Wieland, 2005). Trial designs more typical of diagnostic assays can provide important clinical information regarding their predictive accuracy. Patients and treating oncologists can benefit from chemotherapy sensitivity and resistance assay results obtained from non-interventional studies even with limited findings as supplements to other clinical data when deciding on a treatment.

The poor drug response and survival outcomes for malignant melanoma are very likely related to the heterogeneity of chemosensitivity as well as frequent constitutive resistance to individual cytotoxic drugs. Several mechanisms may account for the phenomenon of resistance that includes failure of the drug to reach and/or affect its intracellular target (Dhar et al., 2003; Dunn et al., 2004; Tas et al., 2005). Tumor cells

display a variety of mechanisms by which they evade immune detection and destruction and render the immune response ineffective.

The development of chemoresistance is a persistent problem during the treatment of local and disseminated disease. Therapeutic agents selectively, but not exclusively, target actively proliferating cells and include at present, DNA alkylating agents, anitmetabolites, intercalating agents and cytokines. Resistance constitutes a lack of response to drug-induced tumor growth inhibition; it may be inherent in a subpopulation of heterogeneous cancer cells or be acquired as a cellular response to drug exposure. Although regulatory approval may require efficacy in as few as 20% of the trial cohorts, a drug may subsequently be used in unselected patients displaying resistance to the treatment (Luqmai, 2005). Principle mechanism may include altered membrane transport via the P-glycoprotein product of the multi-drug resistance (MDR) gene as well as other associated proteins, altered target enzyme (i.e. muted topoisomerase II), decreased drug activation, increased drug degradation due to altered expression of drug-metabolizing enzymes, drug inactivation due to conjugation with increased gluthaione, sub cellular redistribution, drug interaction, enhanced DNA repair or failure to apoptosis as a result of muted cell cycle proteins such as p53 (Senchenkov, Litvak and Cabot,: 2001; Hussein et al., 2003; Tas et al., 2005). Attempts to overcome resistance mainly involve the use of combination drug therapy using different classes of drugs with minimally overlapping toxicities to allow maximal dosages and with narrow cycle intervals necessary for bone marrow recovery.

The combination of genetic instability together with molecular heterogeneity displayed by malignant cells render the construction of effective treatment programs

difficult. A non-trivial problem is the drug development system itself which for reasons of scientific necessity does not assume answers in the short time frame seen in the course of metastatic melanoma. Unraveling the complexities of cellular behavior to intercede in tumor cell proliferation and yield effect tumor response to cytotoxic agents is a therapeutic goal. The complexities of cellular behavior and drug resistance include apoptosis.

The ability to escape suicide (apoptosis) is a hallmark of most cancer cells and often correlates with tumor aggressiveness and resistance to traditional anticancer drug treatments. According to Catherine Denicourt (Science 2004) (Catherine Denicourt of Howard Hughes Medical Institute and Steven F. Dowdy of the Department of Cellular and Molecular Medicine, University of California San Diego School of Medicine, "academic and industrial laboratories are engaged in a Herculean effort to develop new molecules that reactivate the apoptotic program in tumor cells by specifically targeting protein-protein interactions. Modulating or mimicking protein-protein interactions with biologically active peptides or chemical compounds offers an attractive strategy for therapeutic intervention in specific disease pathways." Targeting growth factor receptors within the endothelial cells, for example, with anti-angiogenesis therapies have been shown to affect the apoptotic pathway response. (Dauffenbach L, Torres C, and Fruehauf J American Association for Cancer Research (AACR), 2003 Endothelial Cells Co-Cultured with Breast or Ovarian Cancer Demonstrated Differential Gene Expression and Apoptotic Responses, Abstract #3325). Many types of cancer take advantage of immune modulating activities of cytokines because of their capacity to act on gene expression and

to down-regulate certain immune responses that might destroy cancer cells (Roth, et al., 1983; Ouaissi and Ouaissi, 2005).

A likely reason for ineffective therapy for metastatic melanoma is the lack of specificity for melanoma cells. Biomarker expression as determined by immunohistochemistry is an efficient and effective tool for screening and discovering new targets in melanoma based on outcomes (Fruehauf, Fruehauf J 2004; Krishnansu S, et al, 2005). An example of this are C-Kit expression markers in solid tumors of differing histologies, including carcinomas of the breast, colon, lung (small cell), endometrium, ovary, prostate, and melanoma may be linked to drug resistance and other prognostic factors. In one study (Fruehauf et al., Fruehauf J 2004) DNA alkylators used to treat solid tumors were found to be significantly less active against tumors that expressed CD117. The investigators also found that increased C-Kit expression was significantly associated with increased MDR-1, HER2 and mutant p53 suggesting that C-Kit expression may be linked to drug resistance and other adverse prognostic factors.

A likely reason for ineffective therapy for metastatic melanoma is the lack of specificity for melanoma cells. The utilization of drugs exploiting targets preferentially expressed in melanoma may be found to be beneficial. Kluger et al. (2004) studied a large cohort tissue micro-array and found that drugs that specifically target HER-2/neu were not likely to be useful for the treatment of metastatic melanoma or as adjuvant therapy for melanoma patients at high risk for recurrence. However, Chung et al. (2004) found evidence to support a potential synergistic effect of abnormal HER-2/neu and EGFR and p53 status in the pathogenesis and natural history of lymph node-negative breast carcinoma. Furthermore, these authors found that a combined analysis of multiple

markers may enhance the prognostic capabilities compared with individual markers. This finding has implications for melanoma as its potential targets through combined analysis of multiple markers in malignant melanoma.

Despite the aggressive nature of advanced melanoma there are no standard biological assays in clinical usage that can predict metastasis and few molecular biomarkers have yet to achieve acceptance in the clinical setting. Tissue-based markers evaluated by immunohistochemistry suffer from a high degree of inter- and intra-observer variability due, in part perhaps, to the inadequacy of reproducible assessments of protein expression using traditional immunohistochemistry (Rubin et al., 2004). One recent advance in this field that promises to automate this process is the development of AQUA, a molecular-based method of quantitative assessment of protein expression. This system integrates a set of algorithms that allows for the rapid, automated, continuous, and quantitative analysis of tissue samples, including the separation of tumor from stromal elements and the sub-cellular localization of signals. The methodology and development of AQUA will be discussed in further detail later in this document.

Rimm and colleagues (Hoek et al., 2004) used AQUA to assess global differential gene expression comparing normal human melanocytes with six independent melanoma cell strains from advanced lesions. The data, validated at the protein level for selected genes, confirmed the over-expression in normal cells relative to normal melanocytes of several genes in the growth factor/receptor family that confer growth advantage and metastasis. Some differentially expressed genes reside on chromosomal regions displaying common loss or gain in melanoma or are known to be regulated by CpG promoter methylation. These results provide a comprehensive view of changes in

advanced melanoma relative to normal melanocytes and reveal new targets that can be used in assessing prognosis, staging and therapy of melanoma patients. Berger, Harigopal, Martens, et al. (2003 Appendix A) at Yale Medical School sought to determine if Ki-67 expression correlated with extreme drug resistance in a cohort of 100 melanoma specimens, all tested with the EDR assay at Oncotech Laboratory.

Immunohistochemistry was performed on the tissue micro-array slide with a monoclonal antibody to Ki-67. While there was no relationship between either Ki-67 or EDR and the time to first recurrence, either test may be valuable in terms of predicting response to therapy. Future studies will include this analysis upon collection of treatment and outcome data. Novel targets in melanoma will be discussed in the paper.

The focus of this paper discusses the method of in vitro micro array EDR testing conducted by Oncotech Laboratory Incorporated. This laboratory is located in Orange County California and offers a full line of laboratory tests designed to provide information to oncologists that can be used to tailor treatment to individual patient's cancer, based on the unique biological and genetic properties of the malignant cells obtained from the patient. The tissue arrays performed at this research lab yield results that enable the oncologists and surgeons ordering the tests to stratify their individual patients into good or poor prognostic groups and thus avoid use of specific chemotherapy agents to which the patient's tumor is resistant. The many tests offered at Oncotech will be discussed with emphases given to the Extreme Drug Resistance (EDR) Assay and immunohistochemistry prognostic and predictive marker testing and its relevance to malignant melanoma.

Predictive chemosensitive assays that individualize chemotherapy by drug sensitivity and resistance testing were discovered over two decades ago when a group of scientists raised the possibility that tumor tissue could be cultured with in the laboratory (in vitro) setting in order to yield data about drug response the results of which could be transferable the individual patient (in vivo). Such chemosensitivity assays were challenged and impeded by technical difficulties over the next 25 years. As a result, the in vitro assays maintained attention with limited success in the initial years, lost hold in the mid-1980's, but gained a resurgence of attention and success in the last 15 years. Efficacy and turn-around time for in vitro testing prompted this success.

Presently there is a variety of drug sensitivity and resistance methods and laboratories that conduct them through which surgeons and oncologist can from provide tissue samples for testing. Results from in vitro testing can be provided to the oncologist for utilization in the determination of therapeutic intervention for the individual patient with malignant melanoma. The benefit in assertaining drug response to individual tumor in a laboratory setting prior to administration of chemotherapy for a patient potentially can forestall negative consequences associated with drug administration. Side effects can be deleterious or fatal. The contribution to cost savings can be readily argued. At present use and overuse of medication is of realistic concern as exemplified when considering the state of our society in relation to antibiotic use. A profound example of the implications and conservation of diagnostic testing and use of therapeutic agents is found with antimicrobial prescription determination. The effect of the treatment decision is in large part made for the individual patient, yet the overall effect on the population should not be excluded. The treating clinician is charged with keeping abreast the economic concerns

and consequences of her or his therapeutic decisions. It is for this reason that the treating clinician needs to maintain the ability to decipher the benefits and disadvantages of diagnostic tools that constantly enter into therapeutic choice. The astute clinician continues to utilize accurate tools based on critical evaluation of such tools and the moral obligation to use them when indicated. The same is true when prescribing medication. Practicing medicine does not occur in a vacuum. Moral obligation dictates consideration of therapeutic choice as it affects the individuals within the community of practice as well as the greater community on global scale. Therapeutic choice must balance the individual needs of the patient within the context of the population being treated. There is a compelling need to standardize testing procedures and establish conditions for laboratory detection and accurate identification of disease processes.

Antimicrobial Use on Global Scale and In Vitro Diagnostic Testing Utilization

Efforts that monitor over-treatment with antibiotics are an imperative that face the medical community at large. Effective and accurate in vitro diagnostic testing methods found to identify bacterial organisms are proving advantageous on global scale in these efforts. The discovery and identification of in vitro diagnostic tools which may augment treatment decisions for difficult diseases or diseases with high incidence offer tremendous contribution to the overarching goal of disease management. Evaluations of in vitro diagnostic tool utilization by clinicians which prove accurate diagnostic and rational use of effective therapeutic approaches for disease processes with high rates of morbidity and mortality and cost containment for the populations under treatment will contribute to this goal. Such evaluations are made on a scientific and clinical continuum in order to best identify and clarify their advantage and disadvantage.

The clinical utilization of rapid diagnostic tests for the detection of specific bacterial antigens in serum to supplement therapeutic decision making for acute respiratory infectious (ARI) is proving useful in some developing countries. ARIs are one of the most important causes of morbidity and mortality in children throughout most of the world (MartinsTeixeira, 1999). More than four million children under five years of age are estimated to die from ARI every year. This represents about 30% of the 14.25 million deaths of children under five years of age that occur in the developing world each year (Gwatkin, 1980; World Health Organization, 1983; MartinsTeixeira, 1999). Most of the rapid diagnostic tests have been based on particle agglutination or enzyme-linked immunosorbent assay (ELISA) tests and the utilization of these in vitro diagnostic testing method have been well documented for example in the treatment of acute respiratory

infections (ARI). The success of the ELISA serological tests is limited as they present certain technical difficulties. According to Lu et al. (2005) protein micro array assays present a higher positive rate and sensitivity (86.1% and 1: 2000) compared with traditional ELISA screening methods for SARS and could provide a rapid, parallel and high-throughput antigen screening platform. The discovery of novel diagnostic testing methods and the enhancement of existing methods provide ongoing evidence for disease management. Studies which evaluate test utility identify strengths and weaknesses of the effectiveness, accuracy and economic benefit in the overall improvement of therapeutic intervention. This information can offer direct benefit to the clinicians who treat the diseases and the patients and the population for whom such utilization may benefit.

Choices made about diagnostic test utilization depend on the prevalence of disease and the value placed on increased diagnostic accuracy and efficacy. Risk adjustment assessments which evaluate test utilization consist of a series of techniques that account for the health status of patients when predicting or explaining costs of health care and effectiveness of treatment for defined populations or for evaluating retrospectively the performance of providers who care for them. Inclusion of assessments incorporates patient preference, their perception of treatment and quality of life needs. The clinician treating disease is charged with consideration of the effects of treatment decision on an individual basis as well as its effect on the population. This balance is maintained and enhanced by renewed consideration of diagnostic techniques that improve upon the collective goal to contain disease on global scale and to make it affordable and accessible where possible.

According to a paper published by Bryce et al. (2003), "In 42 countries with 90% of child deaths worldwide in 2000, 63% if these deaths could have been prevented through full implementation of a few known and effective interventions." Health systems are constantly inequitable, providing more and higher quality services to the well-off, which need them less, than to the poor, who are unable to attain them (Gwatkin et al, 2004). According to Gwatkin (2004) the inequities are likely to continue in the absence of concerted efforts to ensure that health systems reach disadvantaged groups more effectively. Gwatkin contends that this situation need not be accepted as inevitable, for there are many promising measures that might be pursued, one of which is use of one or more of the several techniques that seem to be effective in at least some of the settings where they have been tried. Gwatkin adds that the empowerment of people to have a more central role in health system design will also improve the health care system.

There are examples in the literature, as cited later in this document, to indicate that in general patients do prefer to be included in therapeutic decision making. One paper by Tang et al. (2005) examining patient satisfaction with "information exchange" domain from their doctors during the SARS outbreak in Taiwan in 2003, showed patients were most satisfied with understanding their treatment plan about their illness (100%) and doctor being honest about their illness (97%) and being understood regarding their illness (96%). Knowledge is power. The more accurate clinical data available to the treating clinicians the more confidence existing with which the clinician can inform their patient.

Diagnostic Testing and Treatment of *Helicobacter pylori*

This document includes discussion of the value of diagnostic testing methods in the detection of gastric cancer (Sierra et al., 2003). The discussion includes citation from current research as it pertains to therapeutic choice in the setting of limited resources and with regard to economic burden in the treatment of *Helicobacter pylori* (*H. pylori*) and its association with gastric cancer. Studies are cited in this document demonstrating evidence that genetic mutation may prove etiologic in the transformation of cells found in *H. pylori* bacterial disease which result in gastric malignancy. Diagnostic testing to identify the presence of *H. pylori* in humans to augment therapeutic decision attempts to uncover effective methods which can demonstrate accuracy and economic benefit for the patients being treated. Scrutiny as to the utilization of diagnostic testing methods involves modification of treatment regimen based on the population being treated and the needs of the individuals within that population. *H. pylori* and malignant melanoma are no doubt two very different disease processes yet a percentage of the selected challenges inherent to *H. pylori* regarding drug resistance and therefore treatment, may offer insight when discussing the spectrum of therapeutic challenge for malignant melanoma. Lessons learned in view of the challenges in one disease that may be of value in another are discussed. An example of the discussions contained within the document comparing the two diseases and the utilization of diagnostic testing to augment treatment determination follows.

The colonization of the human gastric mucosa with *H. pylori* bacterium invariably results in the development of chronic gastritis and subsets of patients have a progression of the chronic gastritis to either ulcer or cancer (Malfertheir et al., 2005). Epidemiological

evidence indicates that the proportion of all gastric cancers attributable to *H. pylori* infection, and hence potentially preventable upon elimination of this risk factor, is somewhere in the range of 60-90% (Malfertheir et al., 2005). This portends significant benefit in terms of morbidity and mortality, not least in populations with high prevalence of *H. pylori* infection coupled with high incidence of gastric cancer. The utilization of the C-urea breath test after unsuccessful treatment of *H. pylori* is increasing in clinical practice (Gisbert, 2005). The observation of a pattern of histological (active) gastritis without the concomitant finding of *H. pylori* raises the suspicion of diagnostic error (Gisbert, 2005). Antimicrobial resistance incompletely explains eradication failure in treatment of *H. pylori* (Borody et al., 2002) rather an impaired immune response may contribute to failed eradication after standard therapy. *H. pylori* can induce apoptosis of gastric cancer cells (Chen et al., 2005). The mechanism of process still needs further elucidating but its similarities to the challenges inherent to the behavior of malignant melanoma will be brought forth in this document. The burgeoning problem of drug resistance is salient and is translated to drug choice and treatment determination. These decisions may be made less problematic with the utilization of diagnostic tests if found to be accurate, effective and economic. A discussion of these variables and consideration of the advantages and disadvantages of available diagnostic tests is the specific aim of the research contained within this document as may be beneficial in clinical practice.

In vitro testing may demonstrate economic incentive to medical insurers. If it can be shown that in vitro testing of tumor drug response in the lab yields effective and accurate advantage, then its utilization may in fact translate from the US outward globally. In this way, discovering a test that is may demonstrate economic benefit is a

huge success in the fight against the devastation of a disease like malignant melanoma. Economic concerns are a reality in the U.S. and especially abroad where resources are limited. There are still challenges involved with in vitro testing however its acceptance is increasing. As of September 2000, Medicare began to cover certain of the in vitro testing of tumor tissue in the U.S. (Medicare Newsletter, 2000). Until this coverage, the cost of the testing was absorbed either by the patient or the surgeon and/or oncologist directing therapy. Coverage for single agent testing was approved nationally if that testing occurred in the state of California. Medicare reimbursement for multi-agent testing is under consideration and likely to be approved at this writing. Insurance reimbursement is a factor where economic benefit may be realized as the widespread utilization of in vitro testing assays in the U.S slowly gains momentum.

Evaluation Cohort Study of Drug Resistance in Malignant Melanoma Using EDR In Vitro Testing

The evaluative research for which this document is written was conducted to learn further about utilization of in vitro testing for malignant melanoma and establish cause for ongoing consideration of its utilization in clinical practice for those who treat melanoma. The research examined clinical decision making and in vitro drug testing utilization within a medical community who comprise the Yale Cancer Center Melanoma Unit. The research focused on therapeutic choices and decision making processes made in concert with a multidisciplinary approach of surgical and medical specialists. The research is retrospective and is evaluative in method and involves a small cohort of 78 patients. All of the patients in the study were diagnosed or progressed to stage III or IV melanoma during the years from 1994 through 2000. The patients primarily received medical treatment through Yale University Medical School and Yale New Haven Hospital. Patient histories, progression of disease, physician treatment, tumor tissue drug resistance testing results, and patient decedent dates were collected via medical clinic and hospital medical records, serology and pathology results, physician interview and participation in Yale Cancer Center Melanoma Weekly Conference. The data collection effort provided medical data prior to surgical evaluation and including course of disease through death. By the time of the data collection effort, all of the patients in the study were deceased.

Each patient in the cohort had tumor tissue surgically removed by a surgeon, Dr. Stephan Ariyan. The tumor tissue from each patient was sent by overnight express to Oncotech Laboratory Incorporated for EDR in vitro assay testing. Results from

Oncotech Inc. EDR testing were provided to Dr.Ariyan and to Yale oncologist Dr. Leonard Farber. Dr. Farber was the primary oncologist who oversaw treatment for each patient in the study through to their demise. The specific aim of the research was to obtain understanding and data regarding utilization of the EDR test results provided by Oncotech Lab for the surgeons and oncologists who are members of the Yale Cancer Center Melanoma Unit for their patients who received treatment who diagnosed with stage III and IV malignant melanoma.

The surgeon and oncologists who treated each patient in the study comprise the multi-specialty team at the Yale Melanoma Unit at the Yale Cancer Center. The director of the Yale Cancer Center Melanoma Unit is Dr. Stephan Ariyan who established the Unit in 1976 and continues to hold the same title. Dr. Ariyan is surgeon specializing in the treatment of head and neck cancer, breast cancer, and malignant melanoma. The Yale Cancer Center Melanoma Group meets weekly for clinical conferencing for the purpose of presenting individual patient cases of advanced stage III and IV melanoma, in particular for those patients in whom treatment is difficult or rendering limited progress.

Inclusive to the research presented in this study are observations made during two years of observation of the weekly conference and interviews conducted with its members. Collectively this group of medical experts participates in an exchange of medical expertise with the goal to improve patient outcome through a multi-disciplinary approach. In addition to the presentation of individual patient cases and resultant treatment plans, the members discussed the utilization of the in vitro Oncotech EDR results inclusive to treatment determination. Each member participates in clinical trial to varying degree and discussion of such participation contributes to the conference format.

Each member is involved in a clinical research in addition to the treatment of melanoma. The observations and interviews were conducted to obtain information about the multi-disciplinary approach employed for those patients in the practice with stage III and IV melanoma. The observations and interviews also sought to ascertain data about the utilization of in vitro EDR testing.

The research in this document sought to determine whether the in vitro testing was considered and/or utilized by the surgeons and oncologists in the group and how variables such as drug toxicity, quality of life issues and economic burden of treatment affect patient preference. It sought to determine methodology as to how these variables are executed in decision making for the members of the Yale Cancer Center Melanoma Unit within the context of a multi-disciplinary approach for patients with advanced melanoma who may not respond to conventional therapies. The multi-disciplinary approach can serve as a model which proves very effective for individualized treatment of advancing melanoma where drug resistance is extreme and quality of life issues and economics of treatment are of significant relevance.

Global Practice Measures in Drug Treatment

The research presented in this document contains research conducted in Costa Rica by Drs. Rafaela Sierra on *H. pylori* and Rodrigo Zeledón on Chagas Disease infections, respectively. These diseases are presented in this document as examples of the interface between immunological function and carcinogenesis when faced with drug resistance and clinical treatment determination. A portion of the discussion in this document focuses on the potential for the treatment of these diseases in relation to drug resistance and global clinical practice measures. Treatment approaches to these diseases confers immunologic challenge that can be seen in both diseases in consideration of the utilization of diagnostic tools by treating clinicians. There are similarities in the current challenges that are comparable to that of malignant melanoma especially in regard to diagnostic tool consideration. However, it should be emphasized that the scope of these comparisons is restricted to clinical practice measures and disease management and not specific mechanisms of melanogenesis.

The data collected from Costa Rica, Kenya and Burma is incorporated into this document to discuss drug resistance and immune system evasion strategies in cancer disease and in consideration of containment-strategies in drug prescribing practice and drug testing development and utilization. This research in Kenya was conducted in the summer of 1987, in Burma in the summer of 1998 and in Costa Rica during the years of 2001 through 2004. This data collection offers enhancement to the discussion of the disease management challenges that confront clinical medicine on global scale.

The purpose of the research contained in this thesis is to demonstrate the value of and consideration for utilization of drug resistance testing in malignant melanoma.

The thesis hypotheses are as follows:

(1) Clinicians do not use the results of in vitro testing provided by research laboratories.

(2) If in vitro EDR results are used by the clinician, their use will reduce cost and toxicity to the patient.

(3) If in vitro tissue results are used by the clinician, the patient will benefit through increased progression to survival.

The data collected for this evaluative study which considers the utilization of EDR testing results by physicians in the Yale Cancer Center Melanoma Unit was used concurrently in a study which examined biomarker identification in metastatic melanoma conducted at Yale Medical School by David Rimm and colleagues (Berger, Harigopal and Martens et al., 2003, Appendix A, abstract). The tumor tissue samples surgically obtained from the cohort of patients for both studies was catalogued and preserved on slides at Yale Medical School in the pathology laboratory of Dr. Rimm.

Dr. David Rimm is a Yale Medical School pathologist who developed an automated method technique for analysis of tissue arrays called AQUA: Automated Quantitative Analysis (Dolled-Filhart, Rimm, 2002). The AQUA method allows for systematic pathologic identification and rapid assessment of tissue biopsies. The technique consists of a set of algorithms that provides a reproducible, automated, quantitative analysis of expression for a given biomarker from tissue samples. The AQUA method was used to produce tissue microarray cores using tumor tissue collected from the same patient cohort to assess nuclear antigen Ki-67 correlation with EDR (Berger et al (2003, abstract). Although Ki-67 and extreme drug resistance are both predictors of aggressive tumors, no correlation was found between Ki-67 expression and drug resistance in eight of nine drug assays tested (Berger, Harigopal and Martens et al.,

2003, Appendix A, abstract). The results of the study and relevance for EDR testing will be further discussed in this paper.

What follows is a brief description of the principal conclusions of the study and the remainder of its organization. The evidence from the study did not support the hypothesis that clinicians do not use the results of in vitro testing. In this small cohort sample, it was not proven that the surgeon and oncologist did not utilize the Oncotech Inc. testing results. Based on interviews with the treating surgeon and oncologist and through individual chart review, it was determined that the drug(s) chosen for treatment for the individual patient was shown to be resistant in the EDR assay panel testing results provided by Oncotech. This view would disprove the hypothesis indicating the conclusion that the clinicians did utilize the extreme drug resistance test results to augment treatment determination. Further evaluative studies may substantiate these findings.

In vitro drug resistant testing asserts to avoid direct costs of ineffective therapies and costs of managing treatment related morbidity. Based on the findings of the study, it is reasonable to surmise that cost savings and reduction in morbidity may be incurred through utilization of EDR in vitro testing; however the hypothesis that in vitro EDR results if used by the clinicians will reduce cost and toxicity to the patient was not definitively proven. The summarization that EDR test utilization may provide evidence for economic benefit as well as reduced toxicity to the patient may stimulate further study.

No standard adjuvant therapy has shown increased overall survival in malignant melanoma (Asceirto et al., 2005). The evaluative study conducted did not prove nor

disprove with certainty the hypothesis that utilization of in vitro EDR test results will increase survival for patients with advanced melanoma. However, the findings in the evaluative study does submit that the EDR test results in specific cases may be useful in physician decision making in the treatment of patients with stage III and IV melanoma. Further research is needed to ascertain and secure parameters of accuracy and feasibility of EDR testing for confident utilization in clinical practice for this malignancy.

Beyond this introduction, this paper will further be divided into five chapters. Chapter 1 provides a discussion of the diagnosis, treatment, and epidemiology of malignant melanoma. The chapter discusses drug resistance for malignant melanoma and the challenge in drug treatment determination for the treating clinician. Drug resistance, drug prescribing practices and social responsibility are discussed in the chapter to provoke questions as drug resistance soars at alarming rate on global scale. Chapter 2 delineates the benefits and limitations of extreme drug resistance testing assays. Chapter 3 depicts the research evaluation cohort design, data and results undertaken for the research contained in this thesis. Chapter 4 discusses the multi-disciplinary approach of a heuristic clinical medical model and eventiary medicine for the treatment of malignant melanoma. Observations collected from Yale Cancer Center Melanoma Unit and their relevance is developed herein. Chapter 5 includes a discussion of the current utilization of lymphodepletion and autologous T-cell transfer regimens in evaluating new therapies for malignant melanoma. Chapter 5 further discusses challenges in drug resistance and treatment on global scale. The chapter concludes with a discussion for a proposed model for a melanoma unit using the elements and data defined from both the cohort study and

the observations of the Yale Cancer Center Melanoma Unit as it may stimulate further and provocative research.

The topical elements discussed in this thesis include: drug resistance testing and screening for novel therapeutic agents; the identification of patterns of resistance for melanoma malignancy; patterns of cross-resistance and sensitivity in treatment naïve and relapsing tumors; identification of genomic and proteomic profiles associated with resistance; correlations of in vitro drug response; preclinical in vivo effect; and clinical outcome associated with a therapeutic tailoring of individual chemotherapy treatment regiments whose end-goal is reduced morbidity and increased survival in malignant melanoma.

Various assays are under scientific scrutiny for clinical utility and accuracy to achieve these endpoints, including several in vitro clonogenic and proliferation assays, cell metabolic activity assays, molecular assays that monitor expression of markers for responsiveness, in vivo tumor growth and survival assays in metastatic and orthotopic models, and in vivo imaging assays. Descriptors and the advantages and disadvantages of these assays will be included in this document which will commence following the discussion of melanoma and the drug resistance that beleaguers this malignancy.

CHAPTER 1: MALIGNANT MELANOMA

The incidence of malignant melanoma, its development, diagnosis and staging, along with approaches to current treatment are pertinent and are each considered in this chapter. Cancer survival and incidence statistics provide background measures relevant for clinical practice, scientific research, and clinical trials. These statistics are calculated through database registries that record how many individuals are affected by specific cancers. The next section is an overview of cancer database and general terms that are applicable throughout the document including incidence, prevalence, and mortality. These terms are discussed first in order to clarify the statistics on cancer that are presented subsequently.

International Databases

Databases that catalog incidence and survival for cancers are activated through national registries. The U.S. Congress established the National Program of Cancer Registries (NPRC) by enacting the Cancer Registries Amendment Act, Public Law 102-515, in 1992 and reauthorizing the program in 1998. Congress mandated the Centers for Disease Control and Prevention (CDC) to provide funds to states and territories to improve or enhance existing cancer registries and to set standards for data completeness, timeliness, and quality. As of 2004, the CDC funds a total of 49 statewide and territorial cancer registries including Puerto Rico and the Virgin Islands. These registries cover over 96% of the US population.

Over a million new invasive cancer cases are recorded each year by the NPCR state registries (National Program of Cancer Registries, 2004). In 2000, the CDC established the NPCR-Cancer Surveillance System (NPCR-CSS) to evaluate cancer

incidence data. In 2001, it was determined that malignant neoplasms caused 23% of total deaths in the U.S. and were its second most common cause.

Cancer Incidence Databases

Incidence, by definition, is the number of new cases occurring. Incidence is expressed either as an absolute number of cases per year or as the rate per 100,000 persons on an annual basis. The latter measure approximates the average risk of developing a cancer in one year and is used for comparisons across countries, within areas, and in populations over time. Primary prevention strategies for all cancers (such as reduction in sun exposure for melanoma) aim to reduce incidence but rates often trend upwards due to factors unrelated to those precautions. Also, the introduction of programs for early detection has been observed to result in temporary increase in incidence as sub-clinical cancer cases are discovered (Parkin et al., 2005). This phenomenon has been noted in the early detection of primary cutaneous melanoma and breast cancer.

Mortality is the number of deaths. Correspondingly, the mortality rate is the number of deaths per 100,000 persons on an annual basis. Mortality is the product of the incidence and the fatality of a given cancer. Fatality, the inverse of survival, is the proportion of patients that die. Mortality rates therefore measure the average risk to the population dying from a specific cancer within a specified period (usually one year). Prevalence describes the number of persons with the disease alive at a particular point in time. This next section will describe the agencies that collect and analyze cancer rates (Parkin et al., 2005).

Cancer Statistics

For the last 30 years the International Agency for Research on Cancer has prepared estimates of the global cancer burden. Beginning in 1975, the agency gathered data on the number of new cases for 12 of the common types of cancer in different areas of the world (Parkin et al., 2005) which has now expanded to include 26 types of cancer. Their summaries are made available in the GLOBOCAN data base which categorizes findings by gender and age group.

The global data base is built from estimates of incidence, mortality, and prevalence in each national population. Estimates for 20 world areas, as defined by the United Nations (UN) Populations Division, were obtained by combining age-and sex-specific rates for component countries as a weighted average (Parkin et al., 2005).

Cancer cases where statistics are available from GLOBOCAN 2002 (Parkin et al., 2005), number 10.9 million new cases of cancer, 6.7 million deaths, and 24.6 million persons alive with cancer, within three years of diagnosis. The most commonly diagnosed cancers are lung (1.35 million), breast (1.15 million), and colorectal (1 million). There are 160,000 estimated cases of malignant melanoma. The most prevalent cancer in the world is breast cancer with up to 4.4 million survivors up to five years following diagnosis (Parkin et al., 2005). Among cancers, those most commonly associated with death are lung (1.18 million), stomach (700,000), and liver (598,000).

Cancer prevalence is estimated from incidence and survival. The variation in cancer incidence and prevalence throughout the globe is found to be secondary to geography and the risk factors associated with lifestyle and environment. Statistics on survival from cancer, like its incidence, are also available from cancer registries and are sometimes published in formats that permit comparisons between different centers within

a country (U.S. National Cancer Institutes (NCI) Surveillance, Epidemiology, and End Results Programs (SEER) or between cancer registries in different countries (Mathers et al., 2001; Sant, Aareleid and Berrino, et al. 2003; National Program of Cancer Registries, 2004; Parkin et al., 2005).

Incidence data from registries that participate in the NPCR and SEER are reported to NCI and the CDC and were made available in January 2004. The primary sources of cancer incidence are medical records. Staff at health care facilities abstract cancer incidence data from patients' medical records and send it to the regional or state registry. Both NPCR and SEER registries collect data using uniform code items to facilitate comparisons. Of the cases included in the reports, 93.3% were confirmed by positive microscopic findings (histology, cytology, or unspecified microscopy method).

Cancer Mortality Databases

Cancer mortality data are compiled in accordance with World Health Organization (WHO) regulations which stipulate that member nations classify and code causes of death in accordance with the current revision of the International Classification of Diseases (ICD). The ICD details disease classification and also provides definitions, tabulation lists, the format of death certificate, and the rules for coding cause of death (Mathers et al. 2001; National Program of Cancer Registries, 2004; Parkin et al. 2005).

The SEER program is considered the reference standard for quality among cancer registries around the world. As such, it is the most authoritative source of information on cancer incidence and survival in the US. The SEER statistical model program produces frequencies, rates, and survival statistics for cancer (Mathers et al., 2001; Parkin et al., 2005).

The SEER statistics provide a basis for the description of the variability in cancer incidence and death rates across different populations for the identification of groups that might benefit from evidence-based cancer control measures. Cancer control planners can use these data, for example, to more effectively focus cancer prevention and control activities within racial, ethnic, and geographic populations.

INCIDENCE OF MELANOMA

Melanoma Statistics as Reported

One in every three cancers diagnosed worldwide is a skin cancer (World Health Organization-4, 2005). The incidence of both non-melanoma (BSS and SCC) and melanoma skin cancers has been increasing over the past decades. Currently, between two and three million non-melanoma skin cancers and 132,000 melanoma skin cancers occur globally each year (World Health Organization-4, 2005). According to WHO Skin Cancer Foundation Statistics, one in five North Americans and one in two Australians will develop some form of skin cancer in their lifetime (World Health Organization-3, 2001).

The data from the 2002 GLOBCAN series reveals the age-standardized incidence rates in Northern America to be 16.4% for males and 11.7% for females. Male and female incidence rates vary considerably across countries. For example, in Australia, rates are relatively high: 37.7 % for males and 29.4 % for females with rates in Southern Africa and Central America respectfully quite low respectfully: 2.4% for males, 2.3% for females and in Central America, 1.3% for males and 1.7% for females.

In the U.S in 2004., melanoma is estimated to be the fifth and seventh most common cancer in men and women respectively (Balch et al., 2004) and its incidence is rising faster than any other type of cancer (Urist and Soong, 2004). Since 1973, the overall incidence rate of melanoma in the U.S. has more than doubled from 5.7% to 14.3% (Gentry, 2003). Melanoma accounts for approximately 1-2% of all cancer deaths worldwide (Balch et al., 2004).

The Annual Report to the Nation on the Status of Cancer (Howe et al., 2001) contains statistics on cancer trends in the U.S. The report revealed long-term trends for the four most common cancers (breast, lung, prostate, and colorectal) with an update on all other cancers. The report found from 1992 through 1998 that total cancer rates declined in males and females and that a decline in cancer incidence rates was only seen in males. Overall incidence rates in females increased slightly due to an increased rate of breast cancer occurrences among older age groups.

From 1992 through 1998, the incidence rate of melanoma in the U.S. was found to increase an average of 2.7% per year and 2.9% per year for both white males and white females, respectively. Melanoma is reported as rare among black, A/PI, AI/AN, and Hispanic populations (Howe et al., 2001).

The death rates from non-melanoma forms of skin cancer are low, yet these cancers can cause considerable damage and disfigurement if left untreated. Fortunately, when detected early, approximately 95% of these carcinomas can be cured (World Health Organization-2, 2005). While melanoma accounts for only 5% of skin cancers, it has been shown to cause approximately 75% of skin cancer death (Sladden, 2004). Once diagnosed, there is no method to predict which people will progress to the advanced

form. However, the variation in risk factors, whether through geography or exposure, contributes to the orchestration of programs of prevention and to clinical practice.

Statistics Used in Clinical Practice

Global and national patterns of cancer incidence and mortality are a compilation of real data, extrapolation from representative sampling and informed guesses. The practice of medicine and clinical decision making is achieved through a compilation of evidence-based medicine, extrapolation or rules of thumb (heuristics) based on previous experience and informed guess based on expertise.

Cancer statistics are collated based on averages. Available data can serve as guidelines for clinical practice. These guidelines whether stored in the memory of the clinician, or obtained from databases can be quite useful in clinical practice. The lessons for inferring generalizability are not straightforward, although the implications of variation for decision making depend critically on quality of life, cost-effectiveness thresholds and tumor response. There has been little study of the causes of variation, whether differences in study results among countries are systematic, or whether they are important for decision making (Barbieri et al., 2005). Knowledge of incidence and prevalence can aid diagnostic decision making. Risk assessment based on general statistics must be taken into account when considering the individual patient. The epidemiology of melanoma and risk factors will be described next.

EPIDEMIOLOGY OF MELANOMA

Principles of Melanoma Epidemiology

A thorough review of the epidemiology of melanoma can be found in the Textbook of Surgery, 17th edition in the chapter by Marshall Urist and S-j Song (2004). Here the authors describe melanoma as principally a disease of Caucasians, particularly those of Celtic ancestry. The authors note that melanoma can occur at any age but rarely develops before puberty. The authors found that the incidence of melanoma begins to rise with puberty and increases until 65-70 years of age and then falls, with a significant incidence in the third and forth decade of life with the median age of diagnosis from 45 to 55 years of age.

Little information exists on the epidemiology of melanoma and the role of solar radiation in the development of melanoma in the pigmented populations (Hu et al., 2004). Hispanics and Afro-Americans have a significantly lower incidence of melanoma than Caucasians, with Afro-Americans having the lowest rate of melanoma (Hu et al., 2004). According to a World Health Organization Report (2005) melanoma occurs slightly more often in males than females and the prognosis is slightly better for females when other prognostic factors are taken into consideration. The report states that anatomic distribution of melanoma varies between genders; it arises more commonly in the lower extremity in females and trunk, head and neck for males. These differences in distribution are not accounted for by sun exposure alone yet causation for these differences are not fully understood to date.

The incidence of melanoma under the age of 30 was found to be 3.3% (Borbola et al., 2005) in a study conducted by the Dermatology Department of the National Institutes of Oncology in Budapest from 1993 till 2003. In young adulthood the main risk factors

were the number of atypical nevi and repeated or severe sunburns in childhood. The skin type was also an important risk factor.

According to Wagner and Casciato in their chapter on "Skin Cancers" in The Washington Manual of Clinical Oncology (2002), Caucasian men have 20 to 40 nevi by the third decade of life. Wagner and Casciato describe that nevi continue to form throughout adult life; however, only approximately 1 in 500,000 become malignant. Accordingly, of the types of skin cancers known in humans (basal cell carcinoma, squamous cell carcinoma, and melanoma), melanoma carries the highest morbidity and mortality owing to the fact that it progresses rapidly throughout the body via the bloodstream or the lymphatic system. Both common acquired and congenital nevi without cytologic atypia may progress into dysplastic nevi and many of these lesions are stable, but a few may progress to a malignant melanoma (Clark et al., 1984; Goedegebuure, Liyanage and Eberlein, 2004).

Wagner and Casciato state that melanoma frequently progresses to different organs simultaneously and that patient symptoms are subject to the location of melanoma sites. They site frequent locations of clinically evident metastasis include skin and lymph nodes in 59% of patients, lungs in 3%, liver in 20%, bone in 17% and other sites comprise the remaining 8%. Further data by Wagner and Casciato find that malignant melanomas can arise in any organ of the body including the placenta or fetus of a pregnant female. About five percent of patients with melanoma present with symptoms of distant metastasis without apparent primary cutaneous site. While over half of melanomas develop in normal areas of the skin, they can also develop in mucosal surfaces or at any other site in the human body where neural cells migrate. Cho et al.,

(2005) conducted a study to further the hypothesis that cutaneous melanoma at different anatomic sites develops through divergent pathways but found that age, family history of melanoma, and hair color did not statistically differ by anatomic site of the cancer. The risk to the development of melanoma is discussed in the next sections.

Risk Factors for Melanoma

Exposure to physical or chemical carcinogens are responsible for most cancers in industrialized countries which includes smoking, ingesting alcohol or particular foods, and exposure to sunlight and chemicals. According to Goedegeburne, Liyange and Eberlien (2004) in the chapter on "Surgical Oncology" in the Textbook of Surgery, 17th edition, epidemiologic studies have shown strong correlations between both internal and external factors and cancer, although the exact sequence of events related to its development is still unknown. The authors provide examples of leukemia where internal immunosuppression is associated with lymphomas as are external x-rays and gamma rays. Accordingly, exposures to other external elements that are known chemical carcinogens such as asbestos and polycyclic aromatic hydrocarbons, benzene, and aromatic amine have been associated with lung cancer, leukemia, and bladder cancer. The single most critical determinant of future cancer incidence and mortality in general is the ability to reduce tobacco use in all segments of the population (Gallagher, 2004). Smoking is associated with lung cancer, bladder cancer, and cancer of the mouth, pharynx, larynx, and esophagus.

The major risks for developing cutaneous melanoma are similarly composed of external and internal factors, sun exposure and genetic predisposition (Naldi et al., 2005). Accordingly, relevant risk factors include living geographically in areas with intense

sunlight, having a history of sunburns and frequent sun exposure in childhood or adolescence, and being Caucasian. Sunburn at any age is associated with an increased risk of melanoma as is sporadic overexposure to the sun (Howe et al., 2001; Naldi, et al., 2005). Incidence rates are also greater among persons of higher socio-economic status. This most likely reflects the positive association of sun exposure with an affluent lifestyle and education (Naldi et al, 2005). Relevant risk factors that appear to be associated with genetics fair skin, light-colored eyes, blonde or red hair, numerous nevi, a history of abnormal appearing (dysplastic or atypical nevi) moles, family members with melanoma, or a personal history of melanoma (Naldi et al., 2005). Other melanoma risk factors related to external influences include chemical exposure, physical agents such as radiation and burns, and factors that lead immunosuppression. A discussion of ultraviolet radiation exposure and melanoma follows.

ULTRA VIOLET RADIATION

Induction of Melanoma

Recent increasing trends in melanoma incidence are suggested to reflect increased sunlight exposure in susceptible populations specifically attributed to solar ultraviolet (UV) radiation (Gilchrest et al., 1999; Urist and Soong, 2004; Turgay et al., 2005; World Health Organization-1, 2005). Fears et al. (2002) analyzed lifetime residential sunlight exposure data from 718 non-Hispanic white patients, mid-to later decades, with a diagnosis of invasive cutaneous melanoma. The data was gathered from clinics in Philadelphia and San Francisco and tested against the matched controls of 945 patients from outpatient clinics with similar catchment areas. Individual melanoma risk

associated with average annual UVB flux was strong and consistent for both men and women. The investigators found that the association of melanoma for total adult hours outdoors was notable for men of all skin types and women who develop a suntan. They estimated that 50% to 80% of lifetime summer exposure occurred for those with invasive cutaneous melanoma in their early childhood suggesting an association with the development of melanoma later in life (Fears et al., 2002).

Solar radiation exposure, particularly childhood exposure, is strongly implicated in human melanoma. As ozone layers are depleted, the atmosphere loses more of its protective filter function and more solar UV radiation reaches the Earth's surface. It is estimated that a 10% decrease in ozone levels will result in an additional 300,000 non-melanoma and 45,000 melanoma skin cancer cases (World Health Organization-1, 2005). The global incidence of melanoma continues to increase; however, the main factor that predisposes a person to the development of melanoma seems to be recreational sun exposure and a history of sun tanning.

The spectrum of sunlight that causes melanoma in humans has not been definitely established particularly with respect to the roles of UVB, yet its effects are under study by various groups according un-published research by Noonan et al. (2003) as presented in abstract form at the 2003 First International Melanoma Research Congress in Philadelphia, Pennsylvania (Noonan et al., 2003). According to eleven US cancer registries that constitute the SEER Program melanoma incidence was associated with increased UV index and lower latitude only in non-Hispanic whites according from 1992-2001 (Eide and Weinstock, 2005). A monograph entitled, "Environmental Health Criteria 160 -Ultraviolet Radiation" was published by the WHO, jointly in collaboration

with the United Nations Environment Program and the International Commission on Non-Ionizing Radiation Protection. The monograph was the result of a review of scientific literature and was concerned primarily with the effects of UV radiation exposure on human health and the environment. The draft was subjected to a WHO Task Group for final peer review prior to publication. Such a review is considered timely given the concerns over increasing levels of UV radiation at the surface of the earth resulting from depletion of stratospheric ozone (World Health Organization-1, 2005). INTERSUN, a 2005 global UV radiation project is the WHO's response to the imperative to disseminate information about health and environmental hazards of excessive UV radiation exposure. Further discussion into the suspected mechanisms of the physiologic association between ultraviolet radiation and melanoma follows.

Cellular and Molecular Studies

Ultraviolet radiation is one of the non-ionizing radiations in the electromagnetic spectrum. It lies within a range of wavelengths 100 nm to 400 nm. The short wavelength limit of the UV radiation region is often taken as the boundary between the ionizing radiation spectrum (wavelengths < 100nm) and the non-ionizing spectrum. Ultra violet radiation can be classified into UVA (315-400nm), UVB (280-315nm) and UVC (100-280nm), although other conventions for UVA, UVB, and UVC wave lengths have also been used (World Health Organization-1, 2005). Ultraviolet-A and UVB, cause different patterns of effects in the skin; however, both are considered to be carcinogenic (Brash et al., 1996; Urist and Soong, 2004). According to Noonan et al., (2003 conference abstract) the xiphorous fish model of melanoma implicates both UVB and UVA in melanomagenesis. In the Monodelphis opossum model, UVA initiates focal melanocytic

hyperplasia (Noonan, et al., 2003). Noonan and colleagues recently described a new mouse model for UV-induced melanoma that "strongly recapitulates human disease etiology, in histopathology and in molecular pathogenesis. The neonatally UV irradiated HGF/SF transgenic mouse provides a badly needed platform for assessment of environmental risk factors for melanoma." In their work to date, Noonan and colleagues have shown that HGF/SF transgenic mice that were neonatally exposed to physiologically relevant erythemally weighed UV doses from sources with different spectral outputs and melanoma development. These studies indicate that "UVB is sufficient for melanoma initiation in this model but a role for UVA cannot be completely ruled out at this time. Unequivocal identification of the active waveband(s) responsible for melanoma initiation will have a major effect on considerations of the basic mechanisms of melanomagenesis as well as on prevention strategies and risk assessment." The work of Noonan et al. (2000) suggested that "the HGF/SF transgenic mouse would be useful an experimental model for the determination of the consequences of exposure to various regimens of UV radiation and for elucidation of the mechanism by which the consequences of such are realized." Their work to date further indicate that the dramatic rise in incidence of malignant melanoma experienced by populations both within the US and throughout the world over the last several decades has been attributed to enhanced exposure to the UV spectrum of sunlight radiation.

Ultraviolet-B is thought to induce the effects of sunburn and increases melanin production and is considered the most carcinogenic part of the UV radiation spectrum (Gilcrest et al., 1999; Urist and Soong, 2004). Ultraviolet-A has a deeper level of penetration resulting in dermal connective tissue damage, loss of elasticity, and skin

wrinkling. It is not clear whether it is the total amount of UV radiation exposure or the sequence in which individuals receive UV radiation that leads to the development of melanoma (Gilcrest et al., 1999; Urist and Soong, 2004).

Those who incur severe burns in childhood appear to be at higher risk for development of melanoma years later (Berwick et al., 2005; Borbola et al., 2005). In contrast, according to Urist and Song (2004), those who receive exposure on a regular basis may not be at as high a risk. There is also a role for skin type since individuals who tan easily seem not to be as high of a risk for the development of melanoma even with prolonged exposure. The inconsistencies in this evidence were further studied by Gandini et al (2005).

A systematic revision of the literature was conducted by Gandini et al. (2005) in order to undertake a comprehensive meta-analysis of all published observational studies on melanoma. An extensive analysis of the inconsistencies and variability in the estimates was performed to provide some clues about the epidemiology of melanoma. Following their systematic review of the literature, the relative risk for sun exposure was extracted from 57 studies published before September 2002. Intermittent sun exposure and sunburn history were shown to play considerable roles as risk factors for melanoma, whereas a high occupational sun exposure seemed to be inversely associated to melanoma. The country of study and adjustment of the estimates adjust for the phenotype and photo-type (P = 0.024, 0.003 and 0.030, respectively). For chronic sun exposure, inclusion of controls with dermatological diseases and latitude resulted in significantly different data (P = 0.05 and 0.031, respectively). Latitude was also shown to be important (P = 0.031) for a history of sunburn, studies conducted at higher latitudes

presented higher risks for a history of sunburns. Role of country, inclusion of controls with dermatological diseases and other study features seemed to suggest, according to the investigators, that "well conducted" studies supported the intermittent sun exposure hypothesis: a positive association for intermittent sun exposure and an inverse association with a high continuous pattern of sun exposure.

Human blonde and red hair appears to be more susceptible to melanoma and the source of their skin and hair pigmentation actually magnifies the damaging effects of UV rays (Takeuchi et al., 2004). This pathway appears to be dependent upon the color of a person's particular melanin. According to Seiji Takeuchi at Yale University Comprehensive Cancer Center, Yale School of Medicine, and others, (2004), melanin protects the skin against DNA damage induced by direct absorption of sunlight's UV radiation. Yet, irradiating melanin in vitro or in cultured cells also generates active oxygen species such as superoxide, which can indirectly induce oxidative base lesions and DNA strand breaks. The investigators found in murine studies, that photosensitization is greater for pheomelanin (yellow and red melanin) than for eumelanin (brown and black). These investigators concluded that UV-irradiated melanin, particularly pheomelanin, photosensitizes adjacent cells to caspase-3 independent apoptosis, and that this occurs at a frequency greater than the apoptosis induced by direct DNA absorption of UV radiation (Tekautz et al., 2005; El-Deiry, 2005)). The in vivo photosensitizing ability of melanin is unknown; however, melanin-induced apoptosis may contribute to the increased sensitivity of individuals with blonde and red hair to sunburn and skin cancer (Takeuchi, 2004).

Other researchers have considered the relationship of lifetime exposure to melanoma development although quantification has been difficult. Hallberg and Johansson et al. (2004) report that intermittent exposure as with sun-related holidays once or more per year is strongly associated with melanoma development; whereas, chronic exposure has in some instances even been found to be protective. They also found that the increased incidence and mortality of melanoma cannot solely be explained by increased exposure to UV radiation from the sun and they contend that the continuous disturbance of cell repair mechanisms by body-resonant electromagnetic fields seems to amplify the carcinogenic effects resulting from cell damage caused by UV radiation.

The degree of damage that UV radiation produces in the skin depends on the incident intensity, wavelength content (UVA or UVB), and depth of penetration of those wavelengths into the skin (World Health Organization-1, 2005). Although UVC is very efficiently absorbed by nucleic acids, the overlying dead layers of skin absorb the radiation to such a degree that there is only mild erythema, and usually no late sequelae, even after repeated exposures (World Health Organization-1, 2005). Much less is known about the biologic effects of UVA.

To produce any change, UV radiation must be absorbed by a biological molecule (World Health Organization-1, 2005). This involves absorption of a single photon by the molecule and the production of an excitable state in which one electron of the absorbing molecule is raised to a higher energy level. The primary products caused by UV radiation exposure are generally reactive species or free radicals which form extremely quickly but which can produce effects that can last for hours, days or even years. DNA is the most critical target for damage by UVB and UVC and while a considerable amount of

knowledge is available concerning the interaction of UV radiation with nucleic acids, controversy still exists as to which lesion constitutes the most important type of pre-mutagenic damage (World Health Organization-1, 2005). UV radiation can induce acute and chronic photobiologic reactions in the absence of exogenous chromophores. Nuclear DNA is a major chromophore to initiate UV-induced physiologic reactions. According to Horio et al. (2005) the XPA-gene deficient mouse, an animal model of xeroderma pigmentosum, develops increased photobiologic reactions including acute inflammation, immunosuppression and skin cancers, because of the defect in the excision of repair of UV-induced DNA lesions.

About 30 years ago, the discovery of the connection between UV radiation and the immune system triggered the field of photoimmunology (Schade et al., 2005). In that time, many aspects were studied, and a complex picture emerged. UV absorption results in multi-tiered molecular and cellular UV radiation-induced events, eventually affecting the immune system. The shorter wavelengths of the UV spectrum (UVB) appear to the most critical players for impairing immune reactions (Schade et al., 2005).

Further there is evidence (according to Schade et al., 2005) that appear to be primary efferent molecular events following energy absorption of UVB radiation, ending with the various afferent cellular changes, such as induction of regulatory T cells. Regulatory T cells and the role of cancer therapeutics for malignant melanoma will receive greater attention later in this document.

Studies of DNA repair defective disorders have established a link between UV induced DNA damage on skin and various types of cancer (World Health Organization-1, 2005). Cell death (apoptosis), chromosomal changes, mutation and morphological

transformations have been observed after UV exposure of prokaryotic and eukaryotic cells. Many different genes and several viruses (including HIV) are activated by UV radiation exposure. The genes activated by UVB and UVC appear different from those activated by UVA.

Exacerbations in sub-erythemal doses of UV radiation have been shown to exacerbate a variety of infections in rodent models (World Health Organization-1, 2005) UV radiation affects infections both at the site of exposure and at distant sites. Recent work indicates that systemic infections without skin involvement may be affected. Enhanced susceptibility appears to result from helper T-cell activity. Suppression of these immune responses appears to be mediated by release of soluble mediators from UVB exposed skin which alters the antigen presentation by Langerhans and other cells so they fail to activate TH1 cells. The resulting immune suppression is antigen specific, can occur regardless of whether or not antigen is applied at the site of exposure, and is relatively long lasting.

Chronic skin changes due to UV consist of skin cancer (both melanoma and non-melanotic), benign abnormalities of melanocytes (freckles, melanocytic nevi or solar or senile lentigines), and a range of other chronic injuries result from chronic exposure to keratinocytes, blood vessels and fibrous tissue. The much increased rates of skin cancer in patients with xeroderma pignentosum, who have a deficiency in the capacity to repair UV-induced DNA damage, suggest that direct UV damage of the DNA may be a step in the cause of these cancers (World Health Organization-1, 2005). This suggestion has also been supported by the observation of UV radiation-specific mutations of the p53 tumor suppressor gene in a proportion of patients with non-melanoma skin cancer. Oxidative

and immune suppressor effects may also contribute to the capacity of UV radiation to cause skin cancer.

Exposure to UV radiation likely plays a key role in melanomagenesis as well as yet unknown factors (Berking et al., 2003). The first critical step of melanoma development, the uncontrolled proliferation of melanocytes, may indeed be induced buy a combination of UV damage and imbalance of growth factor production by cells in the immediate area of the melanocyte (Berking et al. 2001)

UV radiation is a well-known physiological stimulus to melanocyte function and its tanning reaction. The DNA damage introduced by UV radiation was found to be a critical event in skin photocarcinogenesis. Berking et al. (2003) found that normal human melanocytes forced in the laboratory setting to yield an increase in the expression of three growth factors (basic fibroblast growth factor, stem cell factor, and endothelin-3), when combined with exposure to UVB irradiation, transformed to melanoma within four weeks.

In their study (Berking et al., 2003), increased cutaneous expression of these growth factors was achieved by intra-dermal injection of adenoviral vectors containing the respective genes into human skin grafted to immunodeficiency-diseased mice. Of 79 human skin grafts that received the combination treatment of genetic material and UVB irradiation, melanoma was found in 34%. The lesions were positive for biomarkers S100, HMB45, Melan-A and NKIC3. Melanocytic cells isolated and cultured from these lesions formed colonies in soft agar indicating anchorage-independent growth. Flow cytometry analysis revealed expression of melanoma antigens. This is the first report of human cancer induction in which malignant transformation of normal human cells in vivo

was achieved by a combination of natural environmental factors while each factor alone has no such carcinogenic potential.

In another study (Takeuchi et al., 2004), two groups of mice were irradiated. One of the groups had been engineered with pigmentation for yellow or black hair. The second group was composed of albino mice devoid of pigmentation. The mice were irradiated with the same UV rays that penetrate through the ozone layer affecting humans. Cell death was concentrated around hair follicles which are the only location of melanin in mice. Dying cells were pronounced in the yellow-haired mice and absent in albinos. The investigators concluded that while it seems that melanin does filter UV rays, the melanin in hair follicles and particularly in light hair actually increases their damaging effects and causes cell death in the hair follicle (Takeuchi et al., 2004).

The issue of the relative impacts of UVA and UVB exposure is being researched by scientists at the George Washington University Medical School in their study of the Xiphorous fish model in as of yet unpublished work (Noonan et al., 2004). According to Noonan and colleagues in their work presented at the First International Melanoma Research Congress in June 2003, the investigators predicted unequivocal identification of the active waveband(s) responsible for melanoma initiation and their major effect on considerations of the basic mechanisms of melanogenesis as well as on prevention strategies and risk assessment. Although exposure to excessive amounts of solar radiation appears to be the main etiologic factor and has received a good amount of attention in clinical trial, genetic predisposition to melanoma has not been particularly well studied but is currently receiving more attention in scientific research. The

following section offers some current understanding as to genetic risk factors for melanoma as discussed next.

GENETIC PREDISPOSITION AND ALTERATIONS IN MELANOMA

Role for Cell Cycle Proteins in Melanocyte Transformation

Melanoma incidence varies with family history, phenotype and environmental exposures (Stahl, et al., 2004). Several genes may be involved in the biology of melanoma. Both inherited and acquired genetic defects can lead to any neoplastic event. While a family history of melanoma and exposure to ultraviolet irradiation have been known for years as risk factors in melanoma development, the precise genes involved in inherited predisposition were defined only in the last decade.

Germ line mutations in two genes that play a pivotal role in controlling cell cycle and cell division—p16 CDKN 2A (located in chromosome 9p21) and cyclin-dependent kinase 4 (CDK4)—have been detected in autosomal, dominant, high penetrance familial melanoma cases (Stahl et al., 2004). This gene encodes proteins that function by blocking the cell-cycle progression. Mutations in the p16 gene have been demonstrated in melanoma in up to 25% of specimens and in other tumors, such as leukemia, lymphomas, head and neck cancer, and pancreas (Cuevas and Whitman, 2002). CDKN2A and CDK4 have been detected in autosomal, dominant, high penetrance familial cases (Stahl et al., 2004). CDK4 may be involved in a small percentage of familial melanoma patients and has been found to be encoded a protein on the pRb cell-cycle control pathway (Cuevas and Whitman, 2002). In addition to these two highly penetrance genes, germ line mutations and polymorphisms in a few low penetrance genes have been reported in

familial cases: melanocortin-1 receptor, epidermal growth factor, glutathione s-transferase M1, cytochrome p450 debrisoquine hydroxylase locua (CYP2D6) and vitamin D receptor (Stahl et al., 2004).

The Melanoma Genetics Consortium (Newton Bishop and Bishop, 2005) continue to study the genetic epidemiology of the CDKN2A locus. Melanoma families appear to have a dominate mode of inheritance with incomplete penetrance. Members of these families tend to exhibit melanoma at younger ages and have a higher incidence of multiple primary tumors than others. Ocular and/or cutaneous melanoma occurs in several members of these families. Clusters of familial melanoma appear to serve as validation of the etiology of genetics in melanoma. According to Pollock et al, (1998) in the first study that indicated common founders in melanoma families from different continents, germ-line mutations in CDKN2A were shown to predispose to cutaneous malignant melanoma.

Newton-Bishop et al. (2003) studied melanoma within families in the United Kingdom and found that susceptibility is determined by the high penetrance genes CDKN2A, CDK4 and p14ARF. These investigators found that the likelihood of identifying a mutation in CDKN2A within melanoma families was much greater in families with large numbers of affected family members. In the study, 58% of families with four or more melanoma cases had CDKN2A mutations but only 15% of families with two or three melanoma cases were found to have the mutation. They concluded that in most CDK2A families in the UK, the predisposition was found only in melanoma cancer. Valid estimates of the risk of non-melanoma cases in CDKN2A families are not available at this time.

The Melanoma Genetics Consortium has collaborated to produce estimates of the penetrance in CDKN2A which show some variation with latitude, being higher in areas of UV exposure such as Australia. In other cases, they found that low penetrance susceptibility genes such as MC1R explain familial clustering particularly in populations of fair skinned individuals living in areas with intense sunlight. Family clustering occurs in about one percent of cases in the UK but 10% of cases in Australia, illustrating the putative interaction between genes related to susceptibility and the environment. According to Newton-Bishop et al. (2003), other putative low penetrance susceptibility genes remain to be identified or investigated further such as polymorphisms in the EDF gene.

The most common genetic determinants of skin cancer are the genes that control skin color so that the genes expressed as black skin are protective (Newton-Bishop and Bishop, 2005). The genetic instability of cancer cells seems to play a large role in cancer development. However, scientists have disagreed for nearly a century about whether genomic instability such as that caused by DNA repair defects are the "starting gun" for the development of cancer or merely the result of it. A recent article in *The Journal of Nature Genetics* (Grimm, 2004) provides, "the strongest evidence yet for the starting gun theory." The article describes the concept of the identification of altered mutations in the genes involved and number of chromosomes affected that result in childhood cancer. Grimm (2004) writes that, "It is widely agreed upon that almost all cancer cells have gained or lost entire chromosomes."

The Genes and Environment in Melanoma Study (GEM Study) is population-based, international study of melanoma now in its fourth year (Berwick et al., 2004). The

study is focusing on genetic pathways and melanoma, in particular the relative roles of DNA repair, the cell cycle, and the melanocortin receptor. It is anticipated this study and others like it will yield more specific evidence regarding genes, mutations and melanoma.

A discussion of melanoma is not complete without inclusion of prevention measures and the next section will discuss some of the current research in the area of prevention and public health policy. Public health officials and medical professionals continue to issue warnings to people about the dangers of UV radiation from the sun, tanning beds and sun lamps (Turgay et al. 2005). It can be anticipated that further understanding of the genetic basis of melanoma, the immunobiology of the disease and prevention strategies may help curtail progression of the disease.

PREVENTION OF MELANOMA

Primary and Secondary Prevention of Melanoma

Skin cancer is more common than any other type of cancer and exposure to the sun is known to contribute to all three types, namely basal-cell, squamous-cell carcinoma and cutaneous melanoma. Primary and secondary prevention has been shown to lower incidence rates of melanoma (Howe et al., 2001). The primary prevention of melanoma involves the avoidance of sun and the reduction of exposure to other risk factors. Secondary prevention depends on the careful physical examination and biopsy of all suspicious skin lesions.

The use of sunscreens is an effective strategy which may be substituted for sun avoidance as a method of primary prevention. However to date, the evidence suggests one would best pursue sun avoidance. Some preparations do not absorb the longer

wavelength UVA effectively. Moreover, some have been found to contain ingredients that are mutagenic in sunlight (World Health Organization-1, 2005). According to a report published with the WHO (2005), ninety percent of cutaneous melanoma (CMM), BCC and SCC are thought to be due to sun exposure. Chemical sunscreens have assumed a major importance in skin cancer prevention initiatives especially for CMM.

There have been 17 case-controlled studies of CMM and sunscreen use published in the literature although relatively few of them actually collected information on the frequency of application, quantity used, and duration of use. In general, studies conducted in light skin white populations showed less protective effect than was originally thought from sunscreen use, and in some cases showed an increased risk among users. Studies in Mediterranean populations suggest a protective effect from sunscreen use. The weight of evidence from retrospective case-control studies suggests that sunscreens may not protect against CMM, and that their use may actually cause risk (Gallagher, 2004). These studies were found to be lacking adequate data to formulate firm conclusions.

In retrospective studies of sunscreen use and skin cancer, serious potential problems resulting from uncontrolled confounding due to host susceptibility were present in the studies. In addition, earlier studies rarely collected sufficient data to adequately measure sunscreen use. Sunscreens of adequate SPF have appeared only fairly recently. Due to these factors it is not possible to draw conclusions about whether sunscreens can protect against CMM. Fewer studies have examined the relationship between sunscreen and BCC or SCC risk. The limited evidence to date, largely from randomized clinical trials, is that sunscreens offer protection from SCC and actinic keratosis, but not from

BCC. For the time being, the use of broad spectrum UVB and UVA protective sunscreens seems to be the best choice. Future studies examining sunscreens both with adequate SPF and controls for confounding data may further demonstrate the benefits and/or risks of chemical sunscreens. Until that time, the best strategy for primary prevention appears to be sun avoidance. With increasing levels of solar UV radiation resulting from the depletion of the ozone layer and the continuing rise of melanoma worldwide, people should become more aware of their UV radiation exposure and take appropriate precaution.

On March 17, 2005 the WHO highlighted that sunbed use poses a risk to skin cancer, and that no person under 18 years of age should use a sunbed. It is shown that young people who get burned from exposure to UV will have a greater risk of developing melanoma later in life, and recent studies demonstrate the direct link between the use of sunbeds and cancer. Some of the sunbeds have the capacity to emit levels of UV radiation that is many times stronger than the midday summer sun in most countries. At present, only a few countries have regulations on sunbeds or their use. Belgium, France and Sweden have legislation limiting the maximum proportion of UVB (the most dangerous component of UV radiation). In France the regulations for UV-emitting appliances are to be declared by the health authority, minors under the age of 18 are banned their use, trained personnel must supervise all commercial establishments and advertisement of health benefits is forbidden. In the U.S., the state of California prohibits anyone under age 18 from using sunbeds or tanning salons. The WHO encourages countries to formulate and reinforce laws in order to better regulate the use of sunbeds and to ban all unsupervised sunbed operations (World Health Organization-5, 2005).

Efforts to educate the public about the potential consequences of melanoma and its prevention may help reduce the number of people who develop melanoma. Reducing morbidity and mortality may be achieved not only by clinical intervention but also through personal responsibility, public education and health policy.

Public Health Policy and Melanoma

Public health policy and funding directed at populations with increased melanoma incidence is a prudent public health prevention measure. Citing Australia as an example, where incidence rates of melanoma remain the highest in the world, melanoma-specific prevention programs have been in place for more than 20 years (Howe et al., 2001). These programs seek to influence individual behavior while altering the social environment both physically and through increased foresight regarding policy. Examples that target individual behaviors are public awareness notices which advise the use of protective clothing and sunscreens to limit sun exposure and others that directly question the desirability of a suntan. Examples of changing the social environment or policy include increasing shade in public areas, planting trees and constructing canopies on playgrounds, reducing workplace exposure, avoidance of midday scheduling of sports activities, and providing inexpensive sunscreen without sales tax. The programs in Australia provide an excellent example of policy and funding directed at sound and effective public health prevention tools. As these policies are aimed at the best primary prevention measure available, they are demonstrative of a country taking steps toward education and reducing risk for melanoma. The Australian policies also provide a useful set of concrete policies other countries might consider adopting. Public health policy and

epidemiology are avenues that may certainly influence public awareness and ultimately reduce the incidence of melanoma.

CLINICAL FEATURES OF MELANOMA

Transformation of Melanocytes

Melanoma commonly presents as a changing pigmented skin lesion. Signs of advanced melanoma include nevi which ulcerate, bleed, and itch or contain internal angular indentation or notches, or that are absent of skin within its borders. The development of additional satellite nevi proximal to the original nevi can be indicative of advanced disease (Sun and Schuchter, 2001). Not all melanomas are pigmented. Amelanotic melanoma may be mistaken for undifferentiated carcinoma. Their atypical appearance, from normal pigmentation to pink or purple discoloration, frequently leads to a delay in diagnosis and therefore a poorer prognosis (Urist and Soong, 2004). The next section describes the characteristics of melanocytes.

Melanocytes are the specialized skin cells that arise in the lower part of the epidermis or neural crest where melanin is synthesized. Melanocytes are cells of a neural crest origin that migrate during fetal development to multiple sites in the body, and as such they are prone to metastasize widely and to unusual sites. Most cutaneous melanomas are believed to arise from the basal lamina (Corona, et al., 1996, Hofmann-Wellenhof et al., 2002). Positioned along the basement membrane at the dermal-epidermal junction, these cells are exposed to carcinogenic stimuli that result in malignant transformation to become melanoma. This event is relatively rare compared to the transformation rate for neighboring basal keratinocytes that become BCC and SCC.

Individuals with a history of melanoma are at risk themselves for developing another primary melanoma or multiple other primary melanomas (Balch et al., 2000). Approximately five percent of patients with a primary cutaneous melanoma have or will develop another primary melanoma or multiple primary melanomas (Balch et al., 2001). Approximately 70% of patients with melanoma have had a preexisting nevus at the primary tumor site. De novo melanomas not associated with previously observed skin lesions occur in approximately 30% of patients (Balch et al., 2001). Congenital nevi may engender increased risk for melanoma. Giant congenital nevi have an extremely high incidence of malignant transformation.

All cancerous tumors are characterized by an uncontrolled growth of transformed cells. Tumors do not arise spontaneously; however once triggered, the proliferation of the tumor is independent of the stimulus. The stages of defining the progression pathway are atypical nevi, the precursor lesions and risk markers of melanoma, melanoma in situ and melanoma in the radical growth phase (RGP), which represent the early stages of melanoma, and primary melanoma in the vertical growth phase (VGP) and melanoma in the metastatic growth phase (MGP), which are the advanced stages of the disease (Yang and Becker, 2000). Unlike cells obtained from VGP and MGP melanoma, which can be established in cell lines, cells derived from atypical nevi, melanoma in situ, and RGP melanoma cannot be propagated in vitro. Thus, information regarding molecular markers that may be differentially expressed in the early versus the advanced stages of melanoma can only be obtained from the analyses of specimens.

Activation of telomerase and deregulation of apoptosis contributes to the pathogenesis of a significant number of human malignancies including melanoma (Danial

and Korsmeyer, 2004). Yang and Becker (2000) conducted a study using nevus and melanoma specimens to determine which stage in the pathway of melanoma progression telomerase activity is detected. The investigators found telomerase activity in small percentage yet not all MGP melanomas and not in any of the preceding pathological stages indicating that there is no apparent imbalance between pro- and anti-apoptotic markers in telomerase-positive MGP melanomas compared to telomerase-negative nevi and telomerase-negative VGP and MGP melanomas.

The transformation of melanocytes into malignant melanoma can be divided into five histopathologically and clinically identifiable steps (Clark, 1986; Goedegebuure et al., 2004): (1) common acquired and congenital nevi without cytologic atypia may progress into dysplastic nevi with clear atypical histologic and cytologic features (step 2). Most of these lesions are stable, but a few may progress to a malignant melanoma that tends to grow along the radius of the plaque, the radial growth phase (step 3). Within the plaque, a nodule develops of fast-growing cells that expand in a vertical direction, vertical growth phase (step 4), and ultimately invades the dermis and elevating the epidermis after which the tumor metastasizes (step 5).

Primary melanomas evolve from melanocytes or from precursor lesions through the radial and vertical growth phases. The superficial spreading and lentigo malignant melanomas grow horizontally along the lamina (radial growth phase) before penetrating the deep skin structures (vertical growth phase). The radial growth phase may last as long as twenty years in lentigo maligna and five years in superficial spreading melanoma. In vertical growth phase, the melanoma is associated with invasion of dermal blood and lymphatic vessels. Nodular melanomas have this vertical phase from the onset. Local

lymphatic spread results in satellite nodules of melanoma appearing near the site of the primary tumor (satellitosis). Draining lymph nodes are frequently involved after the vertical growth phase develops.

The prognosis of radial growth phase is excellent irrespective of thickness or other variables. Curable radial growth phase melanomas can be recognized by surveillance of patients identified by screening for risk markers which include dysplastic nevi, common nevi, freckles, and other indicators of chronic or acute sun exposure or sun sensitivity. The prognosis in the vertical growth phase depends on attributes of the tumor and of the host. The most powerful predictors of survival are tumor mitotic rate, presence of host tumor-infiltrating lymphocytes (TIL) within the vertical phase, and tumor thickness.

Two-thirds of patients who develop clinical metastases following treatment of a primary cutaneous melanoma initially present with loco-regional metastases and one-third initially present with distant metastases (Meier et al., 2002). The most important factor in metastatic development according to Meier et al., from a study of 3001 patients is location of the primary tumor irrespective of the metastatic pathway. This finding suggests that for patients with in-transit, satellite metastasis or regional lymph node metastasis, hematogenic metastatic spread had already taken place.

When metastatic disease is suspected, confirmation is facilitated through excisional biopsy, fine-needle aspiration, or core biopsy. Considerable variability exists in the clinical concept and management of melanoma in situ. According to Charles et al. (2005) the true nature of the evolution of melanoma in situ is unknown. Charles and colleagues concluded through evaluation of 1200 dermatologists randomly selected from

the American Board of Medical Specialists Directory of Board Certified Medical Specialists, which no consensus existed regarding appropriate surgical margins or depth of excision for melanoma in situ. The authors recommend surgical margins and depth of excision need standardization d to guide dermatologists in management of the disease and in prevention of further disease. Routine staining of the pathology slides plus immunohistochemical staining with S100, HMB-45, and Melin-A may confirm the diagnosis and differentiate it from other malignancies.

Full staging work-up includes MRI of the brain and CT scans of the chest, abdomen, and pelvis. PET scans can be useful to augment the findings from the CT scans. The patients with bony symptoms undergo radiographs or bone scans. The treatment for melanoma is discussed in further sections of this document under sections of surgical management of primary melanoma and determinants of treatment planning.

Metastasis in Melanoma

The most common sites of metastasis are the lungs, distant subcutaneous tissue, and distant lymph nodes, with isolated lung development occurring in 1.9 to 11% of the patients. Harpole and coworkers (Hofmann-Wellenhof et al. 2002; Fidler et al, 2004) conducted a study of 945 patients who underwent pulmonary metastasectomy, each had nodular or acral lentinous lesions and high Clark level. Findings revealed thicker primary tumors were significantly associated with pulmonary metastasis. The overall five-year survival for patients with pulmonary metastasis from melanoma spread was four percent. Histological type (nodular and acral lentiginous lesions), high Clark level, and thicker primary tumors were significantly associated with pulmonary metastases.

The brain is the third most frequent site of metastases (Eedy, 2003; Hofmann et al., 2005). Brain metastases confer a poor prognosis. Hofmann and colleagues have analyzed efficacy and toxicity of temozolomide with excellent CNS penetration and known activity in 35 patients with unresectable melanoma brain metastasis. The results (unpublished to date) demonstrated that temozolamide may prolong survival when combined with radiotherapy in patients with malignant melanoma. Overall, chemotherapy has not been found to have a significant role in brain metastasis in patients with malignant melanoma. Brain metastases develop in up to half of patients with advanced melanoma, and in 15-20% of the patients the brain is the first site of relapse. Operable solitary metastasis may be excised, but prolonged survival is rare. The treatment options depend on the number and location of the lesions (Eedy, 2003). Steroids may reduce the accompanying swelling and provide palliation. In asymptomatic patients with a central nervous system (CNS) lesion and in patients without major neurologic impairment surgical resection or gamma knife radiation should be considered. Whole-brain radiotherapy has limited success when used as a sole treatment modality.

Patients who previously responded to systemic immunotherapy then relapse with intracranial disease show significant benefit following resection (Eedy, 2003). The benefits of craniotomy include palliation and potential for prolonged disease-free survival. Brain metastases may be treated by sterotactic radio-surgery when limited to fewer than three lesions. This treatment can achieve high rates of loco-regional control of metastases (Eedy, 2003). Douglas and Margolin (2002) and Eedy (2003) found that instillation of chemotherapeutic agents or immune effector cells into the post-surgery cavity may slow disease progression.

Melanoma tends to metastasize to the gastrointestinal tract where it may cause bleeding, intussusception, or obstruction. The characteristic "bull's eye" appearance on contrast studies of small bowel lesions is highly suggestive of melanoma. Patients with obstruction or uncontrolled bleeding from an apparently isolated intestinal lesion may temporarily benefit from resection of the tumor (Urist and Soong, 2004). Pulmonary metastases from melanoma are rarely beneficially resected even if they appear to be solitary.

Choroidal melanomas of the eye were treated historically by enucleation. Small tumors have been successfully treated with high-dose irradiation and local surgical measures. This treatment avoids removal of the eye. Radiation therapy is occasionally useful as a primary or adjunctive modality for treating melanoma patients who are debilitated or who refuse surgery. The most common primary intraocular malignancy in adult Caucasians is intraocular melanoma and its incidence has been stable for many years in contrast to cutaneous melanoma (Ligget and Sears). Uveal melanoma accounts for 12% of all melanomas. It occurs eight times more often in whites than in blacks; patients are usually middle-aged or older. Roughly half of patients with uveal melanoma die from metastatic disease within 10 15 years after diagnosis and enuncleation.

Cardiac metastases frequently occur with melanoma and can occasionally result in arrhythmia or rupture. Antemortem diagnosis is difficult in the absence of malignant pericardial effusion. Patients should be treated with appropriate antiarrythmic agents. Radiation therapy to the heart is probably of little benefit.

Noncutaneous melanomas deserve special consideration because the site of the tumor affects the treatment approach of the primary lesion and the lymph nodes.

Mucosal melanomas compose approximately four percent of all melanomas. They occur more frequently in Asians and blacks, and usually have a poorer prognosis. Staging mucosal melanomas is critical. Excision of the primary lesion may not necessarily be radical because of esthetic and functional deformity. Frequently this type of melanoma initially has lymph node involvement. Metastatic disease is not curable, and it is treated like metastatic cutaneous melanoma although biologically it appears to be a different disease (Urist and Soong, 2005).

Metastatic melanoma originating from an unknown primary site accounts for four to five percent of all cases (Cuevas and Whitman, 2002). These sites include the soles of the feet especially the thumbs and large toes. The percentage of melanomas occurring on the skin is approximately 91%, 5.2% in the eye, and 1.3% on the mucosal surface. Ocular and cutaneous melanomas have several common histologic features, yet their clinical course is quite different. Melanomas occurring in the eye itself have been divided into a variety of histologic types which have different prognoses. The most recent Tumor Node Metastasis Classification (TNM6) combines into each stage tumor categories with similar instead of different prognosis (Kujala and Kivela, 2005). Ocular melanoma rarely metastasizes to lymph nodes since the uveal tract has no lymphatic vessels. Ocular melanoma can develop in the choroids, ciliary body, or uvea and have a peculiar tendency of metastasizing to the liver, sometimes many years after diagnosis of the primary site, giving rise to the syndrome of hepatomegaly, unilateral scleral icterus, and a prosthetic eye.

The treatment options for patients with small ocular melanomas include observation, local treatment, and enucleating. Observation should be considered in

elderly or severely ill patients and cases where a patient has only one useful eye, especially if the tumor is growing slowly. Specific local treatment alternatives include photocoagulation, local resection (local sclerochoroioretinal resection, iridocyclectomy), and radiation therapy (external-beam radiation with photons, stereotactic radiosurgery, brachytherapy, and hyperthermia). Locally advanced tumors or fast-growing tumors may require enucleation.

Melanomas rarely arise in the palate or gingival. The anus and vulva are also potential sites for development of melanomas. Five percent to 10% of vulvar cancers are melanomas even though the vulva accounts for less than 2% of the body surface area (Wagner and Casciato, 2002). Internal sites with a melanocyte population, such as the foregut or central nervous system, may on rare occasion be the site of a primary melanoma.

Melanoma presents as a nodal or distant metastasis as first evidence of the disease in less than two percent of the cases and less than five percent of all cases that present with metastatic melanoma (Chang, Karnell and Menck, 1998; Urist and Soong, 2004). For some patients, the occult primary site may have disappeared spontaneously or may have been excised or cauterized years before the appearance of the metastases. At the advanced stage of metastases, patients usually also have lymphatic metastases. In the case of a lymph node metastasis, a complete regional lymph node dissection is performed on the assumption that it is a regional node and therefore represents stage III, rather than stage IV disease (Schlagenhauff, Stroebel, Ellwanger et al., 1997). The surgical management of melanoma takes into consideration the nature of the tumor as well migratory paths of the disease within the body.

SURGICAL MANAGEMENT OF PRIMARY MELANOMA

Surgical Treatment Modalities for Melanoma

A change in a pre-existing lesion or nevus will often prompt the patient to seek medical attention and evaluation. If all lesions excised and biopsied are negative for melanoma, monthly self-examination to monitor the atypical moles for future malignant changes with interim physician examination is indicated. Some atypical nevi may ultimately develop into a melanoma despite removal of a major portion of the lesion (Sun and Schucter, 2001). Lifetime physician follow-up is recommended for those patients with Atypical Melanoma Syndrome (AMS) or dysplastic nevus syndrome (DNS).

Shave biopsies should be avoided as they compromise the pathologist's ability to stage the cancer adequately (Balch et al., 2004; Sladden et al., 2004). Shave biopsies should be reserved for benign lesions; however, a benign appearing lesion may indeed be early melanoma. An improper shave biopsy may result in a pathology report showing extension of the tumor to the deep margins of the excision. Since the most important factor for prognostics is tumor thickness, this could lead to inaccuracy of the pathologist interpretation which could ultimately result in incorrect decisions regarding wide local excision (WLE), sentinel lymph node biopsy, and adjuvant therapy.

The risks of death from cutaneous melanoma are related to the thickness of the tumor, presence or absence of tumor ulceration and micro-deposits of melanoma in sentinel lymph nodes (Balch, Soong, Gershenwald, et al., 2000; Balch, Buzaid, Soong, et al,. 2001; Thomas et al., 2004), site of the tumor, and the gender of the patient (Elder and Murphy, 1991 and Thomas et al., 2004). Further discussion about thickness of the tumor

and tumor ulceration will be discussed in the section "Pathology and Staging for Melanoma."

Malignant spread occurs both by lymphatic and hematogenous routes. Micrometastases from primary tumors migrate through cutaneous lymphatics to the regional lymph nodes. Traditionally, wide margins of excision have been used to prevent lymphatic spread, but over the past decade, margins have become smaller following previous trials which suggest that narrower margins are safe (Kaufmann, 2001; Kroon and Neiweg, 2000; Bishop, Corrie, Evans, et al., 2002; Thomas, 2004). The issue remains controversial as inadequate excision margins increase the risk of local recurrence and in-transit metastases both of which are associated with a high mortality rate (Balch, Soong, Smith, et al., 2001; Thomas, 2004). Conversely, unnecessarily large margins of excision are associated with greater morbidity and increased cost (Thomas, 2004).

The fundamental principle in the management of primary melanoma is to resect the tumor and minimize the risk of local recurrence. Historically, the first case of cutaneous melanoma ever treated was by John Hunter in 1787 (Davis and Norris, 1980; Eedy, 2003). In 1907 Dr.William Sampson Handley, a Research Fellow at Middlesex Hospital in London, studied the lymphatic spread of a secondary melanoma on a woman's leg using a methodology similar to that which he used for breast cancer (Eedy, 2003). His recommendation, based on this case, was a wide local excision, removal of two inches of subcutaneous tissue down to the level of muscle fascia, together with radical removal of lymph nodes. His work was published in the Lancet in 1907 set the 'rules' for surgical management of malignant melanoma for 50 years (Eedy, 2004; Urist and Soong, 2004). In 2002 publication of the U.K. guidelines for the management of

cutaneous melanoma (Roberts, Anstey, Barlow, et al., 2002; Eedy, 2003) involved augmentation of those rules and today research continues to improve upon the standards available for use in surgical management of malignant cutaneous melanoma.

The current safety margin is based primarily upon the Consensus Conferences (Koh, 1991; National Institutes of Health Consensus Conference, 1992; Serrano-Ortega et al., 2003). The studies of Veronesi and Cascinelli (2003) and Balch et al. (2003) are the only ones to have established, through multiple center studies, the adequate safety margins for each case as dependent on tumor thickness (Veronesi and Cascinelli, 1991; Balch, Urist, Karakousis, et al., 1993; Serrano-Ortega., 2003). The general recommendation of 1.0 cm surgical margin for melanomas less than 1 mm in depth is based upon these studies. According to the NIH Consensus Conference (Koh, 1991; Serrano-Ortega, 2003) the excised block should be taken to the underlying muscle fascia.

The members of the Melanoma Committee of the National Comprehensive Cancer Network, a consortium of oncologists from the NCI-designated cancer centers, annually update their consensus-based guidelines for the treatment of cancer. The 2004 updated guidelines found in Table 1 represent data from four randomized studies testing whether narrow margins or excision could achieve the same results as wide margins.

Table 1 Recommended Margins for Surgical Resection of Primary Melanoma (Urist and Soong, 2004)

Tumor Thickness (mm)	Margin Radius (cm)
In situ	0.5
<1.0	1.0
1-2	2.0
>2.0	≥2.0

The first trial (WHO Melanoma Study) (Cascinelli, Morabito, Santinami, et al., 1998; Urist and Soong, 2004), published in 1991, compared wide local excision (WLE) using a 1-cm margin versus a 3-cm margin in patients with primary tumors < 2mm in thickness (Veronesi and Cascinelli, 1991; Urist and Soong, 2004). The trial included 612 patients, with all local recurrences occurring in the group of patients undergoing a 1-cm radius of excision for tumors measuring 1.1 to 2 mm in thickness. The overall survival for all major groups and subgroups showed no differences. According to Urist al. (2004) these findings confirm that melanomas measuring 1 mm or less in diameter can be resected with an equally low risk of recurrence when 2-cm margin is used. Melanomas between 1 and 2 mm in thickness have an equally low risk of local recurrence when a 2-cm margin is used. The authors state that these margins can be lowered to 1 cm when using a primary closure to the wound. The authors contend that while a narrower margin may result in a small increase in the number of patients who develop local recurrence, there is no difference in overall survival rates.

The second trial in the study was conducted by the Melanoma Intergroup comparing margins of 2 versus 4 cm for patients whose tumors measured 1 to 4 mm in thickness (Balch, et al., 2001; Urist and Soong, 2004). This was a prospective randomized trial with 462 patients with melanoma of the trunk or proximal extremities who received either a 2- or 4-cm radius of excision. After a median follow-up of 10 years the incidence of local recurrence was the same for both groups (2.1% versus 2.6%). The investigators assert the single most important factor that correlates with local recurrence is primary tumor ulceration. Additionally, the investigators advise patients with tumors 1-4 mm in thickness receive WLE with a 2-cm margin.

The third and fourth trials in the study were conducted by the Swedish and French Melanoma Trial groups (Balch, et al., 2001; Cohn-Cedermark, Rutquist, Anderson, et al., 2000; Urist and Soong, 2004). The authors conclude that for melanomas > 4 mm in thickness there is no advantage to resections > 2 cm (Heaton, Sussman, Gershenwald, et al., 1998 and Urist and Soong, 2004). Radical margins of excision of ≥ 5 cm or more confer no survival benefit (Piepkorn and Barnhill, 1996; McKenna et al., 2004).

Veronesi et al., (1988) indicate that "the very low rate of local recurrences indicate that narrow excision is a safe and effective procedure for such patients." These investigators conducted a randomized prospective study assessing efficacy of narrow (1-cm) margin excision for primary melanomas ≤ 2mm. The investigators assessed 612 patients (narrow excision performed on 305 patients and wide excision on 307 patients) with well balanced major prognostic criteria in both groups. The subsequent development of metastatic disease involving regional nodes and distant organs was not different in the two groups (Corsetti, Allen, and Wanebo, 2000). Disease-free and overall survival rates were also similar in the two groups and only three patients had a local recurrence as a first relapse. All had undergone narrow excision, and each had a primary melanoma with a thickness of ≥ 1 cm.

Thomas et el. (2004) conducted a randomized clinical trial comparing 1-cm and 3-cm margins in 900 patients and found that a 1-cm margin of excision was associated with a significantly increased risk of loco-regional recurrences. There were 168 loco-regional recurrences (as first events) in the group with 1-cm margins of excisions, as compared with 142 in the group with 3-cm margins. There were 128 deaths attributable

to melanoma in the group with 1-cm margins as compared with 105 in the group with 3-cm margins. Overall survival was similar in the two groups.

With the aim of regulating the surgical treatment of early tumors, the Guidelines Committee of the American Academy of Dermatology (Sober, et al., 2001; Serrano-Ortega, 2003) established surgical margins of 0.5cm for in site melanoma and histological confirmation that the margins are free of tumor cells.

Unfortunately, melanomas are often found that have been extirpated with insufficient surgical margins. In these cases, the margins are sometimes histologically tumor-free and sometimes histologically occupied (Serrano-Ortega, 2003). Evidence of residual melanoma cells or melanocytic hyperplasia at the margins is indication for surgical re-excision. This creates problems for the patient due to repeat hospitalizations and the associated costs. This also creates problems in disease management which depends upon proper staging of the disease to initiate therapy.

Patients in whom the margins are adequately managed and who have stage I and II disease have a 5-year survival rate of 90% and 70%, respectively (Karakousis et al., 1995 and Cuevas and Whitman, 2002). Tumor margin is often influenced by site. Large defects may require skin grafting or skin flaps. Moh's micrographic surgery should be considered for facial melanoma and other areas where tissue conservation is desired because of its equivalent cure rate (Wagner and Casciato, 2002).

As the clinical accuracy of melanoma diagnosis can vary from 60% to 90% (Kopf, Mintzis, and Bart, 1975; McKenna et al., 2004) and because the recommended margins of excision depend on the tumor thickness, narrow diagnostic excision biopsy avoids unnecessary or extensive surgery when melanoma is not confirmed and permits

margins of wider excision that are consistent with current recommendations (Roberts, Anstey, Barlow, et al., 2002; Sober, Chuang, Duvic, et al., 2001; McKenna et al., 2004).

To conclude, the surgical treatment of cutaneous melanoma has become increasingly conservative over the years. The goal of surgery for any melanoma of any thickness is to ensure complete excision with histolopathologically confirmed tumor-free margins. Wider local excision has a curative objective based on the assumption that at least in the early stages, melanoma is a localized disease and those potentially malignant melanocytes or micrometastases around the lesion should be removed. Alternatively, it has been suggested that any margin clearing the last cell of primary lesion may be sufficient (Ackerman and Scheiner, 1983; McKenna et al., 2004). Despite several recent studies, the findings of Veronesi et al., (1988) remained basically unchanged.

The next section will discuss general definitions of the histology of melanoma. This will be followed with the inclusion of the 2003 AJCC Staging System for Melanoma for use in clinical practice. The stage of a patient's disease is based on the individual histology as reported by pathologic identification of the tumor tissue. The staging provides a working framework for initiation of the treatment regiment. While the mechanism for histopathology and the system of staging provide descriptors of tumor tissue and categorization of patients, there is considerable heterogeneity in each category. This is particularly true as the histology report itself is dependent upon judgments of the pathologist. The next section also discusses the complexity of the diagnostic process associated with each patient prior to initiation of therapy. While staging provides a convenient and somewhat theoretical method of categorizing patients, considerable physician judgment is clearly necessary in the formulation of treatment plans.

HISTOPATHOLOGY AND THE STAGING SYSTEM FOR MELANOMA

Clinico-Pathological Cellular Subtypes of Melanoma

This section discusses the clinico-pathological cellular subtypes of malignant melanoma. The terms should only be considered of descriptive historic interest as they do not have independent prognostic or therapeutic significance (National Cancer Institute, 2002). The interpretation amongst pathologists is considerable.

One study found discordance on the diagnosis of melanoma versus benign lesions in 37 of 140 cases examined by a panel of experienced dermopathologists (Leo, Cagini, Rocmans, et al., 2000 and National Cancer Institute, 2002). To reduce the possibility of misdiagnosis, a group review with the surgeon(s), oncologist(s) and pathologist(s) may enhance the pathology, reading and therefore the treatment plan. Review of this nature was practiced and observed within the Yale Cancer Center Melanoma Group as will be described in Chapter 3.

The histopathologic types of cutaneous melanoma are based on growth pattern and location (Wagner and Casciato 2001, National Cancer Institute, 2002):

> **Superficial Spreading Melanoma (SSM)**, *is the most common type of melanoma, representing about 70% of all cutaneous melanomas. It is more common in women and is most frequently located on the back. The lesion is a pigmented macule or a barely palpable plaque with variegated colors (black, tan, red, brown, or white). Irregularity of the margins, especially the presence of a notch, is a suspicious feature. Slow progression occurs, usually over years before rapid growth and diagnosis. SSM frequently arise from a nevus and spreads in a radial fashion (horizontal spread). These tumors mostly manifest radial growth but eventually enter a vertical growth phase.*
> **Nodular Melanoma (NM)**, *is the second most common type of melanoma and represents approximately 10% to 15% of all cutaneous melanomas. It has more aggressive presentation than SSM, commonly arises in uninvolved skin, and spreads in a vertical fashion (invasive fashion). NM occurs more frequently in men. Most of these lesions are jet-black of dark blue with a distinct border.*

Occasionally, no pigment is present, and electron microscopy or special tissue stains are needed to determine the diagnosis. These tumors grow rapidly and vertically from the onset.

Lentgio Maligna Melanoma (LMM) *represents approximately less than 10% of cutaneous melanomas. It is seen in the elderly in sun-exposed areas such as the head, neck, and upper extremities, and has no sexual predilection. The lesion appears as a large flat, tan-to-black macule of up to four centimeters in diameter, developing in sun-exposed areas of older, light-skinned people, most commonly on the face and neck. The in situ lentigo malgna lesion (or Hutchinson's freckle) shows a horizontal growth phase for up to twenty years and eventually a vertical growth phase anywhere in the involved area. The vertical phase resembles superficial spreading melanoma rather than nodular melanoma.*

Acral Lentiginous Melanoma (ALM) *frequently occurs in patients with pigmented skin, and in neurotropic melanoma. Unclassified melanomas are rare, but desmoplastic may occur around the palms, fingers, nail beds, soles, toes, and mucus membranes.*

Desmoplastic Melanoma (DM) *and unclassified melanomas are rare but do exist. DM mostly occurs in the elderly, and appears as a thick indurated plaque or nodule. DM is frequently amelanotic and is related to neurotropic melanoma.*

Estimates of prognosis are modified by gender and anatomic site as well as by clinical and histologic evaluation. The histologic evaluation by the pathologist is utilized in staging the patient's disease state for purposes of treatment and prognosis. The discussion of the staging system includes recent changes made to reflect the 2003 Melanoma Staging System. The inclusion of the current staging system provides current nomenclature as the system strives to yield uniformity of measure amongst the varying tumor types for diagnostics, prognostics and therapeutics.

The staging system provides a system to stratify findings into standardization and classification taxonomy. The treating physician utilizes this classification to help guide in diagnostics and treatment and from which to communicate findings to additional specialists involved in the management of the patient. The determination for treatment and the decisions that effect the individual's tumor status and needs still rests with the treating physician. Additional indicators such as in vitro test and biomarker results

together with the determinations made through presentation of a patient's individual circumstance to an interdisciplinary team model such as the Yale Cancer Center Melanoma Group may augment the ultimate treatment plan. The next section explains the staging system for melanoma.

Staging System for Melanoma

Historically, the classification of melanoma into various histologic types had a role in clinical management. Until the 1960's, invasive melanoma was considered to be a high-risk disease that required extensive local excision of all tumors. With improved understanding of prognostic factors, management has become based primarily on thickness and ulceration. Tumor thickness has been found the strongest predictor of outcome (Balch et al., 2004; Thomas, 2004; Urist and Soong, 2004) as is associated with risk of local recurrence, regional metastases, distant metastases, with depth of tumor and invasion correlating to inverse survival potential (Balch et al., 2001; van Everdingen et al., 2005).

The revised staging system was implemented in 2003 by the Melanoma Staging Committee of the AJCC (table 2) updated from 1997 (Lange, Sharfman, Aloni, et al., 2004). The Melanoma Staging Committee, initially formed in 1998, comprised of experts from relevant medical specialties with inclusion of leadership from major melanoma centers in Northern America, Europe, and Australia to merge prospective data for the purpose of validating the revisions (Balch et al., 2004).

TNM Classification for Melanoma

The AJCC Database consisted of 30,450 patients with melanoma from 13 cancer centers. Of the grouping, 17,600 (58%) yielded information required for validation for

the prognostic-factors multivariate analysis used to predict melanoma outcome to establish criteria for the current evidence-based staging system (table 2, Lange et al., 2004). Of the grouping, 12,837 (73%) had five years of follow-up data available, 8633 (49%) 10 years of follow-up data, and 2485 (14%) had 20 years follow-up data (Lange et al., 2004). The data on these patients were measured against controls from prospective databases from patients who received no adjuvant systemic therapy.

Table 2 AJCC TNM Classification (Lange et al., 2004)

Primary Tumor Classification (T)
Tx: Primary tumor cannot be assessed (shave biopsy or regressed melanoma)
T0: No evidence of primary tumor
Tis: Melanoma in situ
T1: 01-1.0mm thick
- T1a: No ulceration and level II/III
- T1b: No ulceration or level IV/V

T2: 1.01 to 2.0mm thick
- T2a: No ulceration
- T2b: Ulceration

T3: 2.01 to 4.0mm thick
- T3a: No ulceration
- T3b: Ulceration

T4: >4.0mm thick
- T4a: No ulceration
- T4b: Ulceration

Regional Lymph Nodes (N)
N0: No regional metastases detected
NX: Regional nodes cannot be assessed (i.e., previously removed)
N1: Metastasis in one node
- N1a: Micrometastasis (clinically occult)
- N1b: Macrometastasis (clinically apparent)

N2: Metastasis in two or three nodes of in-transit metastases
- N2a: Micrometastases
- N2b: Macrometastases
- N2c: In-transit satellites(s) metastasis-without metastasis in nodes

N3: Metastasis in four or more regional nodes, matted, nodes, or in-transit metastasis or satellite(s), with metastasis in node(s)

Distant Metastatic Melanoma (M)
M0: No detectable evidence of distant metastasis
Mx: Presence or absence of metastasis cannot be assessed
M1: Distant metastasis
- M1a: Skin, subcutaneous tissue, or distant lymph nodes
- M1b: Lung, metastases
- M1c: All other visceral metastases or distant metastases at any site with elevated LDH

The new staging system (table 2) was published in the sixth edition of the AJCC Cancer Staging Manual and received approval from several governing bodies: the International Union Against Cancer/Union Internationale Contre le Cancer TMM Committee (IUAC/UICC), WHO Melanoma Program, and by the European Organization Research and Treatment of Cancer Melanoma Group (EORTCM).

AJCC TNM Category Descriptions

The T category measures thickness of the primary tumor from the top of the granular layer of the epidermis proximal to the basal lamina to the deepest tumor cell at the base of the lesion based on the Breslow thickness criteria (Breslow, 1970). The depth of tumor is measured with an ocular micrometer recorded in millimeters. In 1970 Alexander Breslow empirically recommended the thickness classification for the staging system with the threshold of thickness identified by two categories (T1 and T2) as depicted in the 1997 AJCC staging system version. At the time, breslow depths less than 0.75mm were considered low threshold. The new 2003 staging system defines T category thresholds in even integers (1.0, 2.0, and 4.0 mm) to represent both a statistical "best fit" and to augment clinical decision making and classification of prognostic groups for node-negative (N0) patients (Breslow, 1970; Balch et al, 2001; Urist and Soong, 2004; van Everdingen).

The category alphabetized subset for T staging indicates the presence or absence of ulceration (Ta or Tb) of an intact epidermis overlying a significant portion of the primary lesion (Balch, Wilkerson and Murad, 1980; Balch, et al., 1978; Balch, 2001). Survival rates for those with ulcerated melanoma are lower than those of patients with a

non-ulcerated melanoma of equivalent T category but are remarkably similar to those of patients with a non-ulcerated melanoma of the next highest T category (Balch, 2004).

The third category in the staging system represents level of tumor invasion defined as subcategories of T (i.e., T1, T2, T3, or T4). In 1969, Wallace Clark and associates (Clark, From, Beradino, et al., 1969; Urist and Soong, 2004) described a classification system of melanoma based on extent of tumor invasion relative to the anatomic layers of the skin. Clark level measures invasion, whether confined to the epidermis or deeper into subcutaneous fat, the papillary dermis, reticular dermis, or subcutis. Differences in pathology readings can vary in interpretation of category subsets.

The M category represents distant metastases. Elevated lactate dehydrogenase (LDH) serum values divide M categories into three groups: M1a, M1b, and M1c. The site and number of metastases and elevated LDH are found to be predictive of poor survival (Lange et al., 2004). Patients with distant metastases to the skin, subcutaneous tissue, or distant lymph nodes are categorized as M1a. Patients with metastasis to the lung are categorized as M1b, and have an "intermediate" prognosis when comparing 1-year survival rates (Balch, Soong, Murad, et al., 1981; Barth, Wanek and Morton, 1995; Lange et al., 2004). Patients with metastases to all other visceral sites have a relatively worse prognosis with designation of M1c. When the serum LDH level is elevated above the upper limits of normal at the time of staging, patients are classified as M1c, regardless of the site of the distant metastases. Because the survival differences between the M categories are small, there are no sub-groupings of stage IV melanoma (van Everdingen et al., 2005).

The staging system it utilizes by treating physicians as: 1) a nomenclature of consistent terms based on prognosis, 2) compartmentalization of patients into definable groups with regard to metastatic risk and survival rates, 3) principles for comparisons of treatment results among different centers, and 4) is a helpful tool for clinical decision making (Negrier et al., 2000; Balch et al. 2004; van Everdingen et al., 2005). According to Balch et al. (2004), the newest staging system is defined on evidence-based medicine principles and provides better quality as it is 1) reproducible and applicable to the practical needs of diverse medical disciplines, 2) based on criteria from consistent outcomes of patients treated at multiple institutions from multiple countries, 3) a reflection of the dominant prognostic factors consistently identified in Cox multivariate regression analysis, 4) criteria which is relevant to current clinical practice and regularly incorporated into clinical trials, and 5) uses required data in the medical records which is easily identifiable by tumor registrars who code staging information.

The inclusion of serum biomarkers into the classification staging system for cancer classification ahs not been historically well accepted. The serum LHD is has been considered a predictive independent factor of diminished survival in multivariate analysis and thus is generally accepted into the staging system (Sirrot, Bajorin, Wong et al., 1993; Eton, Legha, Moon et al., 1998; Keilhotz, Conradt, Legha et al., 1998; Deichmann, Nenner, Bock et al., 1999; Azzola, Shaw, Thompson et al., 2003; Morton, Essner and Balch, 2003; Eedy, 2003).

To conclude, the modifications in the new staging version are represented by the inclusion of quantity of positive nodes, whether the nodes are microscopically or grossly positive, qualification of histologic ulceration and distinction of clinical and pathological

staging of regional nodes (Negier, et al., 2000; Balch et al., 2001; Balch et al., 2004; Lange et al., 2004). The next section highlights current perspectives on regional lymph nodes behavior and prognostic significance. Table 3 represents the AJCC pathologic stage grouping.

Table 3 AJCC Pathologic Stage Grouping (Urist and Soong, 2004)

Pathologic Stage	Tumor	Node	Metastasis
0	Tis	N0	M0
IA	T1a	N0	M0
IB	T1b	N0	MO
IB	T2a	N0	M0
IIA	T2b	N0	M0
IIA	T3a	N0	M0
IIB	T3b	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N1a	M0
IIIA	T1-4a	N1a	M0
IIIA	T1-4b	N2a	M0
IIIB	T1-4b	N1a	M0
IIIB	T1-4b	N2a	M0
IIIB	T1-4a	N2b	M0
IIIB	T1-4a	N2b	M0
IIIB	T1-4a/b	N2c	M0
IIIC	T1-4b	N1b	M0
IIIC	T1-4b	N1b	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

REGIONAL LYMPH NODES

Involvement of Regional Lymph Nodes in Metastatic Melanoma

During invasion, tumor cells can easily penetrate small lymphatic vessels and can be passively transported to the lymph. Tumor emboli may be trapped in the first lymph node encountered in their route, or they may bypass regional draining lymph node to form distant nodal metastasis "skip metastasis". Although this phenomenon was recognized by Stephen Paget in 1989, "its implications for treatment were often ignored in the development of surgical approaches to the treatment of cancers." (Fidler and Balch, 1987; Fidler, 2004).

Regional lymph nodes can become enlarged in the area of the primary melanoma as a result of reactive hyperplasia or growth of tumor cells. Whether regional lymph nodes can retain tumor cells and serve as a temporary barrier for cell dissemination has been controversial (Fisher and Fisher, 1971; Fidler, 2004). Patients identified with clinical stage I and II disease are considered to have invasive melanoma based on absence of both nodal and distant metastases. Pathological Stage III patients have pathological evidence of regional metastases, either clinically in the regional lymph nodes or intra-lymphatic metastases manifesting as either satellite or in-transit metastases. Clinical Stage III staging relies on clinical or radiologic assessment of regional lymph nodes. This staging is inherently difficult for the clinician, especially with respect to assessing both the presence and number of metastatic nodes as the size and determination of lymph nodes on physical exam is not an exact science and subtle variations exist amongst individual patients. Palpating very tiny nodes is not easy, and despite clinical expertise, is subjective and variable. The Melanoma Staging Committee therefore made no

subgroup definitions of clinically staged patients with nodal or intra-lymphatic regional metastases. These patients are all categorized as having clinical Stage III disease. Patients with clinical stage IV patients have metastases at some distant site and are not sub-staged (Balch et al., 2004).

Pathological Stage IV patients are patients in whom there is evidence of metastases at one or more distant sites (Essner et al., 1999; Balch et al., 2004). The number of distant metastases has previously been documented as an important prognostic factor, yet this feature has not been incorporated into this staging system (Balch et al., 2004). There is some variability in the use of imaging tests to comprehensively search for distant metastases. Most centers rely on chest radiograph although others do make use of positron emission tomography (PET) scanning and/or computerized tomography (CT scanning). There is at present no standard imaging mechanism which provides complete confidence in the detection of distant visceral involvement.

The regional lymph nodes are the most common site of metastasis of melanoma (Morton et al., 1992; Reintgen et al., 1994; Gershenwald et al., 1999; Lange et al., 2004). Regional lymph nodes have tremendous staging significance and thus immediate implications for treatment (Buzzaid et al., 1997; Balch et al., 2004). Ten-year survival rates for patients with melanoma who have nodal metastases are represented in Table 4.

The number of metastatic nodes is most strongly associated with 10-year survival compared with all other prognostic factors (Balch et al., 2001). According to Balch et al., (2004), the second most significant prognostic feature for patients with lymph node metastases is the tumor burden of nodal metastases.

Table 4 Ten-Year Survival Rates for Stages I and II Melanomas (Urist and Soong, 2004)

Stage	Tumor Ulceration	T-Stage	Approximate 10-Year Survival (%)
IA	No	T1a	90
IB	Yes	T1b	80
IB	No	T2a	80
IIA	Yes	T2b	65
IIA	No	T3a	65
IIB	Yes	T3b	50
IIB	No	T4a	55
IIC	Yes	T4b	35

The stage groupings for pathologic stage III melanoma use four criteria to assign patients with regional metastases to one of four groups, IIIA, IIIB, and IIIC. Patients with pathologic stage IIIA disease have one to three microscopic (clinically occult and detected by sentinel lymph node or elective lymphadenectomy) nodal metastases arising from a nonulcerated melanoma (T1-4aN1aM0and T1-4aN2aM0). The 10-year survival rate for these patients is 60% (McKinnon et al., 2003; Balch et al., 2004).

The definitions used for clinical and pathological staging of stage III disease are more complicated than those used for the other stages because of the need to accommodate advances in staging of lymph node metastases. The marked diversity in the natural history of pathologic stage III is demonstrated by fivefold differences in five-year survival rates for defined sub-stages, ranging from 69% for patients with a nonulcerated melanoma (regardless of thickness) and a single clinically occult nodal metastases (detected by sentinel or elective lymphadenectomy) to a low of 13% for patients with an ulcerated melanoma of any thickness and four or more clinically

apparent nodal metastases documented by therapeutic lymphadenectomy (Balch et al., 2001). Data representing five-year survival in patients with nodal metastases is found in Table 5. Data representing five-year survival for stage III patients with ulceration is found in Table 6.

Table 5 Five-Year Survival Rates in Patients with Melanoma with Nodal Metastases (Balch et al., 2001; Lange et al., 2004)

# POSITIVE NODES	NONULCERATED		ULCERATED	
Microscopic Involvement	% ± SE	Number	%±SE	Number
1	69±3.7	252	52±4.1	17
2-3	63±5.6	130	50±5.7	111
≥4	27±9.3	57	38±8.8	46
Macroscopic Involvement				
1	59±4.7	122	29±5.0	98
2-3	46±5.5	93	25±4.4	109
≥4	27±4.6	109	13±3.5	104

Table 6 Five-Year Survival Rates for Stage III Melanoma Patients (Balch et al. 2001; Urist and Soong, 2004)

Stage	Tumor Ulceration	N-Stage	Approximate 5-Year Survival (%)
IIIA	No	N1a	70
IIIA	No	N2a	60
IIIB	Yes	N1a	55
IIIB	Yes	N2a	50
IIIB	No	N1b	55
IIIB	No	N2b	45
IIIC	Yes	N1b	30
IIIC	Yes	N2b	25
IIIC	Yes	N3	30

Konstadoulakis et al. (2002) sought to identify the prognostic factors for stage III melanoma patients. They compared survival data of patients presenting with stage I and II disease who subsequently developed a regional nodal recurrence. The goal of the investigators was to identify common prognostic factors and to compare the biologic behavior of the two groups. The two groups were examined retrospectively. The first group included 116 patients with stage III diagnosis. Group two included 57 patients with stage I and II melanomas that had a regional lymph node (LN) metastasis diagnosed at least 6 months after surgical treatment of their primary lesion. The age of the patients, number of disease-involved LNs, site of primary lesion and the presence or absence of palpable LNs proved to be significant prognostic factors in the first group. The survival data for the second group was compared to that of the stage III patients. Five-year survival rates were examined starting from the time of diagnosis. Survival in the second group was 47.37% compared with 25.86% in stage III patients; however, this difference was not statistically significant. Patients who present with stage III seem to have a more aggressive phenotype than stage I and II patients who present with recurrent disease in their regional LNs. The investigators concluded that disease behavior is related to the number of disease-involved LNs, the site of the primary lesion and the type of surgical procedure performed, whether elective or therapeutic LND (Konstadoulakis et al., 2002).

In conclusion, the results of the multivariate analysis used to create the new AJCC Staging System for Melanoma demonstrate "1) tumor thickness and ulceration are the most powerful predictors of survival in patients with localized melanoma (Stages I and II), while level had a significant impact only within the subgroup of this melanomas; 2)

the number of metastatic nodes, the tumor burden, and the presence or absence of melanoma ulceration and of intra-lymphatic metastases satellite or in-transit metastases, were the most powerful predictors of survival in patients with nodal metastases (Stage III); and 3) the number and anatomic site of distant metastases and the presence of an elevated LDH were the most significant predictor of survival in patients with distant metastases (Stage IV)" (Balch et al., 2004).

The patients who have nodal involvement should not be considered a homogenous group. As such, the recommendations that they enter into intensive clinical trials should take into account the marked diversity that each tumor pathology result reveals. Stage III patients are sometimes incorrectly assumed to be at uniformly high risk for distant metastases and therefore may be offered very intense forms of systemic therapy. The clinicians who treat the disease must face the challenge inherent to this variability and heterogeneity among tumor type. An understanding of this is imperative when deciding whether to refer a patient to a clinical trial. Those designing clinical trials too, must be aware of the heterogeneity in particular to melanoma. Seemingly small alterations in the staging of the disease can result in large variations in survival and potentially confound research results.

Survival for those with Stage IV melanoma is measured in months not years (Table 7). Only a minority of Stage IV melanoma patients survive beyond one year (Prignano et al., 2002; Balch et al., 2004). The five-year survival rate for stage IV melanoma is only about 5-6% (Gentry, 2003). None of the prognostic features in the analysis could stratify Stage IV patients into subgroups with a median survival separated by more than a few months. In general, the only Stage IV patients who live beyond one

or two years are those with limited disease who have had a complete surgical resection of the distant metastases (Morton et al., 2003; Balch et al., 2004). There are no long term randomized trials that can be conducted to determine the long-term results of a surgical intervention or therapeutic regimen (Balch et al., 2004).

Table 7 Twelve Month Survival Rates for Patients with Distant Metastases (Urist and Soong, 2004)

Stage	Metastatic Site(s)	Approximate 12-month Survival
M1a	Skin, subcutaneous tissue, lymph nodes	60
M1b	Lung	55
M1c	Other visceral sites	40

LYMPH NODE DISSECTION

Sentinel Lymph Nodes

Lymph node dissection (LND) is reserved for patients with clinically proven lymph nodes and is considered a therapeutic node dissection (Mack and McKinnon, 2004).

The ability to stage patients more accurately with sentinel node biopsy technology has markedly changed the understanding of the natural history of melanoma over the last decade (Essner, Conforti, Kelley, et al., 1999; Balch et al., 2004). This new staging technology is seen by some in the field as extremely powerful and it has caused a significant staging relocation in the new 2003 version of the melanoma staging system. Nonetheless, some still question its accuracy and reliability.

The first node or nodes to which the primary melanoma drains are the sentinel lymph nodes (SLN) and are by definition those nodes at highest risk for regional nodal metastatic disease. Through widespread use of sentinel node lymphadenectomy, there has been considerable stage migration of patients previously staged as "node negative," but

who in fact, had undetected nodal metastases (Balch et al., 2004). This is due to the dependence of Stage III classification on lymph node metastasis (Balch et al., 2004).

This next section will give a very brief history of the development of SLN mapping, current state of utilization of the method, its efficacy and controversy related to its use. After finishing this discussion related to staging of the disease, the treatment approaches and problems of drug resistance will follow. The heterogeneity and resultant multi-drug resistance seen in malignant melanoma tumors are discussed in subsequent sections, in particular the section titled "Drug Resistance in Melanoma."

The Multi-center Selective Lymphadenectomy Trial Group Morton et al., 2005) was just published in the Annals of Surgery (Sentinel Node Biopsy for Early-Stage Melanoma" Accuracy and Morbidity in MSLT-I, an International Multi-center Trial.) published recently in the Annals of Surgery (Morton et al., 2005). The conclusion from the center's experience is that "lymphatic mapping/sentinel node biopsy should become standard care for staging the regional lymph nodes of patients with primary cutaneous melanoma."

In the early 1990's, Donald L. Morton MD, medical director and surgeon-in-chief at the John Wayne Cancer Institute in Santa Monica, California, heralded an era in which patients with micrometastases could be identified and staged more accurately, enabling such patients to have therapeutic lymph node dissection, and therefore entered into appropriate adjuvant trials (Eedy 2003; Gentry, 2003). Morton developed the sentinel lymphatic mapping procedure as a means to identify the SLNs. Morton's work was published in a landmark study wherein his promising surgical application for the SLN biopsy was proclaimed.

Prior to this era, the removal of all lymph nodes proximal to the primary cancer site was regarded às standard and essential upon diagnosis of any malignancy. Sentinel lymph node mapping is not an absolute predictive test but one with good yield. Data suggest that SLNs can be identified with success in more than 80% of lymphatic basins dissected (Cuevas and Whitman, 2002; Zapas et al., 2003). Procedurally, technetium 99 radionuclide is injected intradermally at the site of the primary melanoma to facilitate the scanning of all surrounding lymph nodes. Isosulfan blue added to the injected area during the procedure facilitates visualization, allowing the surgeon to remove only the first lymph node (the SLN) into which the fluid flows for testing of melanoma cells. If these sentinel nodes are clear of cancer cells the surgeon has more confidence the cancer has not spread. This method is employed head and neck surgeon Dr. Stephen Ariyan of the Yale Cancer Center Melanoma Unit.

Therapeutic lymph node dissection in the draining lymph node basin in those patients with clinically palpable lymph nodes is well delineated. However, one problem with this procedure is timing. By the time the regional lymph nodes are palpable, the tumor burden is high. The arguments against this procedure are an over-treatment of 80% of patients who are not found to have occult lymph node metastases and are at risk of the 15-50% postoperative morbidity rate associated with lymph node dissection (Eedy, 2003). Opponents of both SLN biopsy and therapeutic lymph node dissection argue that trials have provided insufficient data to suggest that the procedures improve disease-free survival, at least in all patients (Balch et al., 2001; Dessureault, Soong, Gerchenwald, et al., 2001; Belhocine, Pierard, Labrassinne, et al., 2002; Eedy, 2003).

McMasters et al. (2002) examined in-transit SLN (interval SLN) in patients with melanoma to evaluate whether the risk for nodal metastasis, as seen in the interval SLN, occurs with the same frequency as for patients with traditional nodal basin (i.e. cervical, axillary, and inguinal). The investigators found positive interval SLNs likely to be the only site of nodal metastasis. The study concluded that although positive interval SLNs are infrequently identified, they contain metastatic disease at nearly the same frequency as SLNs in cervical, axillary, and inguinal nodal basins. These investigators recommend that detailed postoperative lymphoscintigraphy and meticulous intraoperative searches for interval nodes should be performed on each patient with metastasis.

Lymphatic mapping and sentinel lymphadenopathy is standard to stage regional nodes because it is accurate and minimally morbid (Bleicher et al., 2003); however, its role for thin (≤1.0 melanomas) is not well known. Bleicher et al. (2003) reviewed a database of over 10,000 patients with melanomas ≤ 1.50 mm thick. Five hundred twelve patients underwent lymphatic mapping and those found with tumor-positive sentinel nodes underwent complete dissections. The investigators found a high nodal positivity rate associated with primary melanomas 1.10 to 1.50 mm thick to suggest that lymphatic mapping and sentinel lymphadenectomy is indicated for this group to help stage regional nodes because of its accuracy and minimal morbidity (Bleicher et al., 2003).

Further endorsement is given by the Sunbelt Melanoma Trial Group (Chao, Wond, Ross, et al., 2002; Eedy, 2003). The trial enrolled 1183 patients into their SLN group where micrometastatic disease was determined by histopathology or immunohistochemistry. Interferon alpha-2b was given for patients with either SLN negative or SLN positive status as mandated by the study randomization. Tumor

recurrence at any site for patients with histologically positive SLNs was 15.5%, while for patients with histologically negative SLNs it was 6% (P=0.0001).

These early results from the Sunbelt Melanoma Trial Group show that early regional lymph node recurrence was very uncommon after positive SLN biopsy and completion of lymphadenectomy (Chao et al., 2003; Eedy, 2003). Patients with positive SLN biopsy were found to be more likely to develop both local, in-transit recurrent and distant metastases within a short follow-up period, but long-term follow-up is needed to detect actual difference in recurrence and overall survival (Chao et al., 2003; Eedy, 2003; Urist and Soong, 2004).

This finding was also made from the observation of and interviews with the Yale Cancer Center Melanoma Unit surgeon, Dr. Stephen Aryian and oncologist, Dr. Leonard Farber. Dr.'s Aryian and Farber participated in The Sunbelt Melanoma Trial by collating and supplying data from their patients to the investigators running the trial as discussed in Chapter 3.

Lymphatic mapping and SLN biopsy have almost completely replaced elective lymphadenectomy in the surgical approach to cutaneous melanoma. SLN biopsy is currently recommended for patients with melanoma who clinically have negative lymph nodes. Given the overall excellent prognosis for patients with thin melanoma, most centers offer mapping only to patients with melanomas larger than 1 mm. Several investigators have reported rates of positive SLN in patients with melanoma <1.0 mm thick ranging from 5%-7% (Zapas et al., 2003). Guidelines for the use of SLN biopsy for intermediate thickness (1.0-4.0 mm) are well established. Current indications for SLN biopsy, ideally performed concomitant to the local wide excision (LWE) are a Breslow

depth greater than 0.75 mm and pathologic ulceration (Zapas et al., 2003). Patients with a single microscopic nodal metastasis detected by sentinel node biopsy have a five year survival rate of nearly 70% (Gershenwald et al.; Lange et al., 2004).

Thin melanomas, increasing in prevalence, are considered highly curable when treated with local wide excision alone, and have a reported 5-year disease-free survival rate of 95% to 98% (Bedrosian et al., 2000) as echoed by a National Institute of Health (NIH) consensus specifying tumor thickness of 1 mm representative of low-risk disease (Corsetti et al., 2000). However in patients with lesions smaller than 1 mm, there is a small but nonetheless positive risk of disease recurrence and death. Despite primary lesions that were otherwise assessed as low risk based on multiple clinical and histopathological factors, other pathological and clinical features may therefore play an important role in the determination of risk of recurrence (Bedrosian et al., 2000). Bedrosian et al. (2000) found that the presence of vertical growth phase adversely impacts survival rates in a melanoma 1 mm or smaller.

Thin Clark level III and IV melanomas may be at increased potential risk for metastasis and late recurrence due to dermal lymphatics which are located at the interface of the papillary and reticular dermis. Thin level III and IV melanomas are at increased risk when compared with all thin melanomas (Corsetti et al., 2000). Given the availability of effective adjuvant therapy with alpha interferon for patients with stage II melanoma for treatment in regional and systemic disease coupled with the minimal morbidity of SLN lymphadenectomy, the investigators suggest SLN biopsy for accurate staging and treatment of all patients with thin melanoma found to have high Clark levels. The investigators suggest that the risk of nodal disease may not be accurately predicted

by the use of a multivariate log model that incorporates thickness, mitotic rate, regression tumor-infiltrating lymphocytes, gender, and anatomical site. Thus, they suggest patients with thin lesions having vertical growth phase should be evaluated for SLN biopsy and trials of adjuvant therapy when stage III disease is found (Bedrosian et al., 2000).

In lesions less than 0.76 mm the investigators do not recommend lymphatic mapping and lymphadenectomy because of cost and morbidity. Cost data associated with the procedure is sparse. Bedrosian et al. (2000) found that it added approximately $915 to the surgical and operating room cost and $1,811 to the overall cost of a wide local excision. These costs do not factor in missed time in employment or medical follow-up. The cost must be balanced against its benefit, especially with patients with little risk of nodal metastases.

Current staging systems and available prognostic models imperfectly predict outcomes in melanoma patients presenting with primary disease. Improved outcome prediction will spare patients who demonstrate little chance of metastasis from the burden of unnecessary therapy while allowing selection of those at higher risk for appropriate clinical management and/or participation in clinical trials. These issues are most salient in the approximately 70% of patients who have thin (≤1 mm) primary melanomas. While thin lesions (new AJCC T1 lesions) are associated with a high cure rate, a clinically significant minority are associated with metastatic disease and death. Thin melanomas may develop metastases and thick melanomas may remain focalized for many years (Zapas el al., 2003; Balch et al., 2004; Morton et al., 2005).

Future innovative methods which increase the accuracy of SLN identification and clinical trials that lend opportunity for retrospective analysis are being assessed. The next

great challenge is to develop methods of SLN assessment that are noninvasive yet even more accurate than present methods (Thompson et al., 2004). Techniques such as in vivo proton magnetic resonance spectroscopy hold great promise. Reverse transcriptase-polymerase chain reaction (RT-PRC) detects minute amounts of melanoma in lymph nodes undetectable by routine histopathologic examination. Multi-institutional studies are underway to determine whether or not treatment intervention can decrease recurrence rates and improve overall survival in patients with only RT-PRC evidence of regional nodal disease (Goydos et al., 2003).

The goal of tumor identification lay in the ability to detect, diagnose and treat with as ideal precision as is possible for the individual patient. Another clinical method extremely useful to treating physicians which is showing rapid and promising development in both diagnostic and therapeutic realm of malignant melanoma is the identification of tumor biomarkers in the sequelae of treatment determination. The next section discusses tumor biomarkers in malignant melanoma.

TUMOR PROGRESSION

Serum Biomarkers in Melanoma

The detection of immunohistochemical biomarkers in tumor material or body fluids is showing great promise in the assessment of diagnosis and also may confer poor prognosis in melanoma and help distinguish poorly differentiated amelanotic malignant melanoma from tumors of obscure origin (Rimm et al., 2001; Berger et al., 2003; Bottoni et al., 2003; Parker et al., 2003; Berger et al., 2004; Cloven et al., 2004; DeVita et al., 2004; Freuhauf, Kyshtoobayeva, and Yu, 2004; Kluger et al., 2004).

Biomarkers can be divided into differentiation (lineage) markers, progression (stage) markers, and others which consist of DNA, RNA or protein. Some progression markers have been identified that show a preference for one or a more stages of melanoma development and to target vaccine development but most lack definitive prognostic information (Bottoni et al., 2003; Kounalakis and Goyos, 2005). According to Kounalakis and Goyos, (2005), "the search is on for biomarkers for their use in the diagnosis, staging, prognosis, and management of patients with melanoma."

As with many types of cancer, the hematogenous spread of melanoma is a bad prognostic sign, and many groups are attempting to detect circulating melanoma cells in patients with different stages of melanoma. Some investigators use direct extraction of intact tumor cells from the peripheral blood and other the detection of surrogate markers of circulating melanoma cells, such as MART-1 (Rimm et al. 2001; Berger, Harigopal, Martens, et al., 2003Appendix A, abstract); Berger et al., 2004; Kluger et al., 2004; Willmore-Payne et al., 2005 However, a correlation between the detection of intact melanoma cells in the circulation and prognosis is controversial. Many other biomarkers are under study including those most widely used in clinical applications: S100-beta, melanoma inhibitory activity (MIA), and LDH. S100-beta is more sensitive and HMB-45 is more specific. The use of serum S100-beta has received a fair amount of attention (Eedy, 2003; Ugurel, 2005). Bottoni et al. (2003) point out the value of serum 100-beta protein as a marker for metastatic melanoma. The investigators evaluated 279 patients with cutaneous melanoma stages I through IV (Bottoni et al., 2003; Ugurel 2005). Patients with Stage IV metastatic melanoma were found to have elevated S100-beta serum concentrations that were higher than those in normal controls of patients with less

advanced disease. Moreover, during a follow-up at 48 months, in the patients observed in this study, serum levels of S100-beta protein were observed to increase and decrease with disease progression and regression respectively.

Regular determinations of both S100-beta and MIA levels during follow-up can also be used for early detection of tumor relapse in melanoma patients as increased serum concentrations of these marker proteins are indicative of tumor growth. As with elevated serum levels of LDH, patients with distant metastases from melanoma who present with elevated S100-beta and MIA serum levels have poorer overall survival than do those who have concentrations within normal ranges (Ugurel, 2005).

Close correlations between the concentrations of these serum markers and tumor load have been found and continue under study. These three markers can also be used to monitor the course of disease and therapy outcome in patients with distant metastases. Since there are no marker proteins for melanoma that are not dependent on tumor load, it is not currently possible to forecast the survival of patients who are tumor free after surgery. Serum markers have not been found to be suitable for screening or for the diagnosis of primary melanomas. The use of serological markers has been discussed by Brocjez and Naeyert (2000) regarding S100 and a wide range of other possible serological markers. The authors reviewed the literature and found that most serological markers were elevated only in advanced disease (Brocjez and Naeyart, 2000; Eedy 2003). Many markers may be found to further therapeutic strategies and help patients who could benefit from more aggressive adjuvant therapies. Much progress has been made and preliminary studies have shown promise as has studies examining circulating DNA as a

prognostic and diagnostic marker of disease in melanoma as well (Kounalakis and Goyos, 2005).

Tumor progression is related to the interaction of the tumor cells with their microenvironment. The tumor microenvironment implies the total functional and structural constellation of neoplastic and non-neoplastic cells and extracellular components. Molecules derived from the tumor stroma may exert paracrine effects on the adjacent neoplastic cells, and they may be shed into the circulation. Based on their tissue distribution, molecules derived, in part, from the tumor stroma can also be considered as progression biomarkers (e.g. proteinases and cytokines) as discussed next.

Ki-67 and Additional Serum Markers in Melanoma

Some of the additional serum markers under study that are expressed in primary melanoma as well as the invasiveness and metastatic dissemination of melanoma include Ki-67 (detected by Mib 1), cycline A , cycline D, p35, MMp-2, beta1 and beta3 integrins, osteonectin, the presence of an inflammatory infiltrate and capillary invasion. The Ki-67 nuclear antigen is a cell cycle associated nuclear protein that is present in all the proliferating phases of the cell cycle (G1,S, G2, and M phase) but is absent in G0 (resting cells) (Gerdes et al., 1984). Ki-67 joins the proliferating cell nucleus Ag (PCNA) as an example of cell proliferation and is a target for antibodies. Ki-67 and p53 immunophenotypes, minimal deviation melanoma may represent a distinct entity for evaluating the distinctiveness of minimal deviation melanoma (Chorny et al., 2003). M1B1 is a monoclonal antibody that is able to recognize an epitope to Ki-67 antigen after formalin fixation and paraffin embedding (Key et al., 1992). M1B1-Ki-67 immunoreactivity may provide useful diagnostic and prognostic information in

melanocytic lesions (Steinbech et al., 1996; Kanter et al., 1995; McNutt et al., 1995; Böni et al., 1996; Kanter-Lewensohn et al., 1997; Sparrow et al,. 1998). Expression of these serum markers has been found to be indicative of a poor prognosis (Guerry et al., 2004). In non-Hodgkin's lymphoma Ki-67 is expressed with diffuse aggressive lymphomas and negatively predicts for both response to treatment and curability (McDonnell et al., 1990; Chu and DeVita, 2001).

Two retrospective studies with Ki-67 have examined prognostic models for melanoma (Guerry et al., 2004). Both studies examined biologically-based prognostic models for 10-year metastasis in patients with thin lesion disease. The first study focused on well-established clinical and histological prognostic factors, the second on factors related to dermal proliferation, the step in tumor progression that follows invasion and is associated with metastatic potential. The first study (n=900) used recursive partitioning and cross-validation to develop "prognostic trees" based on clinical and histological factors. In the second study (n=407), primary lesions were assessed by immunohistochemistry, using the monoclonal antibody mib-1 that recognizes the Ki-67 proliferation antigen. The average Ki-67 expression among dermal melanoma cell was 8.5% (8%-9%), while the mean dermal mitotic rate was 0.77 (0.5-1.0). Ki-67 expression, tumorigenicity and mitotic rate were each statistically significantly associated with 10-year metastasis. In the multivariate model that included these three factors, only Ki-67 and tumorigenicity were significant with adjusted odds ratios of 1.9 (1.2-3.1) and 6.2 (1.4-28), respectively. The investigators of both studies suggest that the addition of Ki-67 expression to prognostic models may improve the accuracy of outcome predictions and that the integration of additional biomarkers of processes related to metastasis and its

restraint promise to yield prognostic models that are clinically useful, that are accurate, generalizable, and effective.

Chung et al. (2004) found evidence to support a potential synergistic effect of abnormal HER-2/neu and EGFR and p53 status in the pathogenesis and natural history of lymph node-negative breast carcinoma. Furthermore, these authors fund that a combined analysis of multiple markers may enhance the prognostic capabilities compared with individual markers. This finding has implications for melanoma as its potential targets. In a study by Kluger et al. (2004) the investigators found evidence to suggest that drugs that specifically target HER-2/neu are not likely to be useful for the treatment of metastatic melanoma or as adjuvant therapy for melanoma patients at high risk for recurrence. Therapies directed at specific cellular targets in melanoma are few in number and lack consistency but continue to be studied.

It is well demonstrated that the use of immunohistochemistry staining aides in the detection of melanoma micrometastasis in sentinel lymph nodes, although it remains unclear which is the optimal pathologic protocol for SLN evaluation. One staining technique involves CD117/Kit. CKit is related to a family of transmembrane tyrosine kinase receptors nuclear antigen associated with cell proliferation. Its signaling through the Akt pathway may suppress apoptosis and confer drug resistance.

A number of in vitro assays have been developed to help tailor cancer chemotherapy. The EDR assay from Oncotech exposes cancer cells to extreme levels of drug, and assesses proliferation by H-thymidine incorporation. Fruehauf et al. (2004) at Oncotech, compared 6,313 individual drug results with CD117/Kit expression in 2878 solid tumors of differing histologies, including carcinomas of the breast, colon, lung

(small cell), endometrium, ovary, prostate and melanoma. CKit expression was present in 34% to 76% of the tumor types evaluated. High levels of expression were found in 17% of breast specimens, 16% of colon cases, 14% of small cell carcinomas of the lung, 13% of melanomas, 11% of endometrial carcinomas, 10% of ovarian carcinomas, and in 3% of prostate carcinomas. The investigators found that DNA alkalators such as BCNU, cyclophosphamide, ifossfamide, temozolomide and dacarbazine, were significantly less active against tumors that expressed CD117 ($p<0.0001$). No association was found between in vitro responses to microtubule directed agents or topoisomerase directed agents and CKit expression. These results suggest that CKit expression may differentially affect apoptosis signals triggered by different classes of chemotherapy. Such findings may be relevant when considering combination treatment with Gleevac and other anticancer compounds.

To evaluate if CKit expression might be related to the metastatic process, these same investigators compared CKit expression in primary versus metastatic breast cancer. No significant difference in expression between these sets of cases was found. CKit expression had not been previously evaluated in the context of other known prognostic factors. The investigators compared CKit expression with mutant p53, HER2, EGFR, MDR1 and ER. Increased CKit expression was significantly associated with increased MDR1, HER2 and mutant p53. No association was noted with EGFR or ER expression. These data suggest that CKit expression may be linked to drug resistance and other adverse prognostic factors. The evaluation of CKit expression in solid tumors may have predictive utility if these results can be independently confirmed.

Quantitative Assessment of Protein Expression: AQUA

Despite the aggressive nature of advanced melanoma there are no standard biological assays in clinical usage that can predict metastasis. Other than those that have been discussed, few molecular biomarkers have yet to achieve acceptance in the clinical setting. Tissue-based markers evaluated by immunohistochemistry suffer from a high degree of inter- and intra-observer variability. This may be due, in part to the inadequacy of reproducible assessments of protein expression using traditional and conventional immunohistochemistry. One recent advance in this field that promises to automate this process is the development of AQUA, a molecular-based method of quantitative assessment of protein expression (Rubin et al., 2004; Dolled-Filhart, 2002). This system integrates a set of algorithms that allows for the rapid, automated, continuous, and quantitative analysis of tissue samples, including the separation of tumor from stromal elements and the sub-cellular localization of signals.

The development of the quantitative assessment began with Battifore (1986), who suggested the idea of piecing together 100 or more different tissue specimens arranged into a block of paraffin for high-through put tissue analysis of DNA, called DNA microarrays (Dolled-Filhart, Rimm, 2002). Further, Kononen et al. (1998) developed a manual micrometer-driven arraying device to core histologic blocks and place the cores into recipient array blocks, called tissue microarrays. These tissue microarrays contained up to 1,000 individual cylindrical tissue biopsy cores in a single paraffin "recipient" block that for analysis. The resultant slide allowed simultaneous processing and high-through put analysis of protein, RNA, or DNA expression on hundreds of samples, representing hundreds of patients. In contrast to DNA arrays, in which hundreds or

thousands of genes are evaluated form a single tissue sample or cell line, tissue microarrays allow the examination of a single gene (or gene product) on hundreds or thousands of patients (Dolled-Filhart, Rimm, 2002).

According to Dolled-Filhart (2002), tissue microarrays have several significant advantages over conventional tumor tissue block sectioning and staining. The most compelling advantage they cite is tissue amplification. According to the authors, standard tissue block is usually exhausted after 60-80 cuts resulting in a maximum of 80 assays per tissue block, however taking core biopsies from a tumor block allows for 60-80,000 assays (a 1,000-fold amplification) while still maintaining the histologic integrity of the tumor tissue. Second, the tissue microarray process allows for reproducibility resultant in equal and simultaneous conditions for antigen retrieval and staining reagents, which they contend reduces the slide-to-slide variability that occurs with large cohorts of conventional tissue sections. The investigators further contend that across a population of tumors, a defined section area of a single tissue spot can be representative of the tumor histology and tends to standardize rather than misrepresent expression levels.

Rimm and colleagues (2000) published the first validation study of quantitative assessment of protein (AQUA) at Yale University Medical School. The purpose of the study was to determine how many cores of breast tumor tissue are necessary to represent antigen expression in tumor tissue as would be assessed by a single conventional slide. Individual markers for prognostic value can be based on absolute expression levels or ratios of expression in user defined sub-cellular compartments. The association of the markers is evaluated with clinico-pathologic data, performed using Kaplan Meier

analysis, log-rank, and Cox proportional Hazards multivariate analysis. S100 is used as a mask for melanocytes.

In 2002, Dolled-Filhart and Rimm published the first validation study to determine how many cores of breast tumor tissue are necessary to adequately represent antigen expression in tumor tissue as would be represented by a conventional slide (Camp, Charrette, Rimm, 2000). The investigators constructed a high-through put redundancy array with 38 invasive breast cancer blocks with up to 10 cores from each breast cancer case. Antibodies to HER-2, Ki-67, ER, PR, and cytokeratin were used to test the array by IHC. The results indicated that the staining of two microarray cores was comparable to analysis of the whole tissue section in greater than 95% of cases and in fact, the investigators found that in the majority of cases, one core alone could adequately represent the tumor.

According to Rimm (2002) a long-standing problem in the analysis of tissue samples is the time-constraint and tedious process of histologic separation and pathologist-based review of sections. These two critical factors, says Rimm, inherent non-uniformity in preparation and subjectivity of analysis, have historically limited the statistical rigor and validity of studies involving tissue.

The AQUA method can address the issue of heterogeneity of different areas of the tumor. It also increases processing capacity which will be essential for standardization in the search for potential markers. This methodology holds out great hope for solid tumors like melanoma and these investigators and others at Yale University Medical School have used the AQUA method to test melanoma.

Tissue microarrays are quite versatile (Dolled-Filhart, Rimm, 2002) and may be valuable in melanoma to validate potential tissue markers as prognostic tools for management of malignant melanoma (Berger, Camp, Divito, et al, 2004). This use of tissue microarrays for the analysis of expression of many genes or proteins (AQUA) toward the goal of target discovery is done in a manner analogous to that used for cDNA arrays.

Rimm and colleagues (Hoek et al., 2004) used AQUA to assess global differential gene expression comparing normal human melanocytes with six independent melanoma cell strains from advanced lesions. The data, validated at the protein level for selected genes, confirmed the over-expression in normal cells relative to normal melanocytes of several genes in the growth factor/receptor family that confer growth advantage and metastasis. Some differentially expressed genes reside on chromosomal regions displaying common loss or gain in melanoma or are known to be regulated by CpG promoter methylation. These results provide a comprehensive view of changes in advanced melanoma relative to normal melanocytes and reveal new targets that can be used in assessing prognosis, staging and therapy of melanoma patients.

Berger, Harigopal, Martens, et al. (2003 Appendix A, abstract) at Yale Medical School sought to determine if Ki-67 expression correlated with extreme drug resistance. To test the hypothesis, the investigators determined the relationship between extreme drug resistance and initial recurrence in a cohort of 100 melanoma specimens. All of the melanoma specimens seen in the Department of Pathology were tested with the EDR assay at Oncotech Laboratory during the years of 1995-2002. The corresponding formalin-fixed, paraffin-embedded blocks were collected to produce a tissue micro-array.

The majority of cases were metastatic (92 cases). Data from the EDR assay was available for the following drugs: 5FU, 5FU+Leucovorin (5+L), Carmustine, Cisplatin, Dacarbazine, Doxorubicin, Etoposide, Mitomycin C, and Vinblastine. Imunohistochemistry was performed on the tissue micro-array slide with a monoclonal antibody to Ki-67. The slide was scored for percent positive nuclei. As the distribution of Ki-67 scores and the EDR assay results were in groupings (extreme, intermediate, and low).

Neither the EDR assay results, nor the Ki-67 score, were able to distinguish aggressive from non-aggressive primary melanomas (i.e., time to first recurrence after initial diagnosis). Although Ki-67 and EDR are both predictors of aggressive tumors, no correlation was found in eight of nine drug assays tested. Also, no relationship was found between Ki-67 expression and primary tumor aggressiveness or EDR and primary tumor aggressiveness. However, the specimens examined were predominantly metastatic tumors where high levels of Ki-67 expression are expected. While there was no relationship between either Ki-67 or EDR and the time to first recurrence, either test may be valuable in terms of predicting response to therapy. Future studies will include this analysis upon collection of treatment and outcome data.

Berger et al. (2004) employed the AQUA technology. Using a tissue micro-array cohort of 405 melanoma lesions and 17 normal skin samples, the investigators analyzed expression of HDM2, the human analogue of murine double minute 2 with automated qualitative analysis. The investigators showed that expression levels in the nucleus were significantly higher in primary melanomas than in metastatic lesions. Furthermore, high levels of expression were predictive of better outcomes. This study demonstrates that

quantitative assessment of protein expression is useful in melanoma to validate potential tissue biomarkers and suggests that human homologue of murine double minute 2 may be a valuable prognostic tool for management of malignant melanoma (Berger et al., 2004).

How Far Has Understanding of the Cell Cycle Evolved?

An article entitled "Recycling the Cell Cycle: Cyclins Revisited" (Andrew Murray, molecular and cellular biologist at Harvard University), was selected in 2004 as one of the outstanding papers from the journal *Cell* in their historic 30 year collection to depict how far biology has advanced. Dr. Murray's article appeared in 1984 and noted how little was understood about the cell cycle. Murray pointedly contended that an inappropriate and excessive focus on individual proteins rather than the complexity of the entire cell cycle engine "crimped our knowledge of the cell cycle." Murray cited the mechanism of the engine, the cellular processes it controls, and the signals that regulate it, had directed us away from thinking of the cell cycle as an integrated whole. Murray charged that, "the ease of genetic manipulation in yeasts compared to the difficulty of biochemistry has kept all but an honorable minority of labs from trying to fractionate and reconstitute either the cell cycle engine or the processes it controls." Dr. Murray (1982) concluded that the next ten years would reveal whether (the scientific community) would, "have the commitment for the hard experiments necessary to challenge the current dogma, overturn it where necessary, and move on to a deeper understanding of the cell cycle."

Cell editors asked the authors selected for this prestigious volume to provide current commentary regarding their historic article and its current relevance. Referencing his own critique, Dr. Murray acknowledges that the broad principles of the cell cycle

oscillator are widely known, yet now asserts that the important differences lie between the proteins and when and where they are expressed.

This article is mentioned in this paper to shed light on the expertise from one noted scientist and his critique on our understanding of molecular biology and the cell cycle. While (the scientific community) has come a great distance in its understanding, full disclosure still eludes those involved in research. While numerous agents have been developed that target specific molecules on cancer cells (Kluger et al., 2004), specific studies for drug targets for melanoma are both conflicting and few in number. Next the role of the immune system in malignant melanoma will be identified.

IMMUNOLOGY AND DRUG RESISTANCE

Immunology in Melanoma

Disseminated melanoma is a radiation-and chemotherapy-refractory solid tumor neoplasm and as such, consideration of tumor immunological response is relevant to the discussion of this malignancy. The solid tumors which have received the greatest degree of consideration are gastrointestinal adenocarcinomas (colon and gastric adenocarcinoma), breast cancer, and ovarian cancer (Dahr, Todd, and Byron 2003). There are not as many published studies in the cases of malignant (disseminated or metastatic) melanoma, soft tissue carcinoma, glioblastoma, and squamous cell carcinomas in general. For this reason, this next section includes in the ongoing discussion a description of the problems with drug resistance in melanoma. The discussion touches upon three issues: the intrinsic immunogenicity of metastatic cells,

the ability of the host to recognize and destroy autochthonous tumor cells and the antigenic heterogeneity of the melanoma.

Molecular analysis has provided three major theories for the preferential outgrowth of tumor cells. The first, the growth factor theory, proposes the tumor cells in the blood or lymphatics invade organs at about the same frequency, but only those that find favorable growth conditions multiply. The second theory, the adhesion theory, proposes that endothelial cells lining the blood vessels in certain organs express adhesion molecules that bind tumor cells and permit intravasation. The third theory is that chemokines secreted by the organ can enter the circulation and attract tumor cells that express receptors for the chemokines. For example, melanoma cells express elevated level of the receptors CXCR4, CCR7, and CCR10 compared to normal melanocytes. Lymph nodes, lung, liver, bone marrow, and skin express the highest levels of ligands for these receptors and are the preferred sites for metastatic spread of melanomas (Goedegeburre et al., 2004).

Within the tumor, certain tumor cells may secrete chemokines that attract inflammatory cells. Additionally cells may in turn secrete growth factors, promoting angiogenesis that directly promote tumor growth. In later stages of tumor growth, the tumor cells may start secreting these factors themselves (Goedegebuure et al., 2004).

One of the manifestations in the development of cancer is the failure of the immune surveillance system. The immune system has the capacity to recognize tumor-associated antigens and develop specific T cell responses to those antigens. The ability to intervene and thus enhance the immune system to achieve a beneficial antitumor response remains an area of intense clinical research. Considerable progress has been made in

expanding our knowledge of the targets for an immune response and our knowledge of the full repertoire of cellular and humoral constituents involved in the generation of effective antitumor response (Grimm et al., 2000; Robbins et al, 2002; Murray, 2003; Ouaissi and Ouaissi, 2005; Sakaguchi, 2005).

While there is some evidence that the immune system may itself fight against tumors, when that system is overwhelmed, as with other diseases, therapeutic drugs are used try to combat tumor progression. Just as tumors may be resistant to the natural defenses of the body, so too, the central challenge in chemotherapeutics for malignant melanoma similarly rests in the drug resistance imposed within the system of the cell cycle. Mechanically, the cells and factors involved in tumor growth are similar to those involved in wound healing. Both processes are characterized by a complex network that involves activation and migration of leukocytes to the site of damage. However in wound healing, proliferation of cells and inflammation subside when the tissue is successfully regenerated, but tumor cells maintain their proliferative capacity and as such are wounds that do not heal such as seen in cutaneous ulcerating lesions.

The possible existence of immune surveillance mechanisms that prevent the development of cancer is also supported by the finding that immunodeficient individuals and patients undergoing long-term treatment with immunosuppressive drugs are at greater risk for cancer than the general population. Experimental evidence that directly links immune mechanisms to the defense against cancer comes from classic experiments in which immunized mice rejected chemically induced syngeneic tumors to help determine how tumors evolve (Dunn, Bruce, and Ikeda, et al., 2002; Goedegebuure et al., 2004). The continuous pressure of the immune system in an incompetent host

(immunoediting) has been studied in mouse models (Dahr et al., 2003) and murine tumor models (Dunn Old, and Schreiber, 2004). Provocative correlative data obtained by studying human cancer holds that the immune system not only protects the host against development of primary non-viral cancers but also sculpts tumor immunogenicity.

The last fifteen years have seen a reemergence of interest in cancer surveillance and a broadening of the concept termed immunoediting (Dunn Old, and Schreiber, 2004) thus further study into elimination (cancer immunosurveillance), equilibrium, and escape as a means to hopefully stimulate development of more effective immunotherapeutic approaches to control and/or eliminate human cancers.

Tumor cells can evade immune detection through many mechanisms and recent research has yielded understanding as to some of the mechanics of escape. Clinical investigators are devising strategies to enhance the development of a robust immune response in the tumor-bearing host (active tumor immunity) or alternatively, by the adoptive transfer of activated effector cells or tumor-specific antibodies into the tumor bearing host (passive tumor immunity). Antibodies that recognize tumor-associated antigens can aid in the pathologic diagnosis of cancer and facilitate the staging of cancer in vitro and in vivo and as well as the detection of recurrent cancer (Dahr et al., 2003). A discussion as to the state of current scientific study on adoptive transfer as utilized for malignant melanoma and in vitro and in vivo testing is found in the final chapter.

The identification of the cellular constituents of the immune response and the knowledge of their functions has been aided by the development of monoclonal antibodies created by the immunization of mice against human immune cells. Each monoclonal antibody recognizes a single glycoprotein antigen that reflects the expression

of a unique cell surface receptor. By convention, these receptors and the cells that express them have been assigned cluster designation (CD) by number (e.g., CD3, CD4, CD8). Histological analysis of excised human and animal tumors has demonstrated varying degrees of immune cellular infiltration (lymphocytes and APCs) which suggests the recruitment of these cells in response to neoplastic proliferation. When analyzed, these tumor-infiltrating lymphocytes (TIL) are often found to be specific for the autologous tumor, with little or no activity against unrelated tumor targets. Accompanying this mononuclear infiltration of tumors is the elaboration of various cytokines that are associated with ongoing immune response. T cells that exhibit specific reactivity against autologous tumors have been isolated from patients with melanoma, breast cancer, ovarian cancer, and colorectal cancer (Dahr et al., 2003) and such findings are relevant to novel therapies under study for melanoma (Smith and Cerundolo, 2001; Bystryn 2002; Hersey 2002; Nestle 2002; Sabel and Sondak, 2002; Bystryn and Reynolds, 2005).

A vital issue in immunology is in understanding how the immune system is able to discriminate between self and non-self, inhibiting autoimmune responses but allowing effective immune responses against microbial antigens (Sakaguchi, 2005). The immune system has evolved several mechanisms to institute and sustain unresponsiveness to self antigens (immunological self-tolerance), including physical elimination or functional inactivation of self-reactive lymphocytes (clonal deletion and anergy, respectively).

There is also substantial evidence that T cell-mediated active suppression of self-active T cells is another elemental mechanism of self-tolerance (Shevach, 2000; Coutinho, 2001; Maloy, 2001; Sakaguchi, 2004; Sakaguchi, 2005). The idea that T cells

which negatively control immune responses is not new for immunologists, yet there has been great controversy as to whether they actually constitute a functionally distinct cellular entity and if so, the weight of its importance in controlling immunologic disorders. In recent years, suppressor or regulatory T cells (Treg cells) have gained resurgent interest (Baecher-Allan and Hafler, 2004; Kakaguchi 2004; Sakaguchi 2005). The resurgence of interest is in part due to an enhanced understanding that the normal immune system endogenously produces as a normal cellular constituent a CD4+ T cell subpopulation that is highly specialized for suppressive function and that an abnormality of a number or function of these cells can be a chief cause of autoimmune and other inflammatory diseases in human and animals (Sakaguchi, 2004; Sakaguchi 2005).

Most endogenous CD4+ Treg cells constitutively express the CD25 molecule (IL-2 receptor α chain (Baecher-Allan and Hafler, 2004, Sakaguchi, 2005)). Additionally the CD4+ Treg cells specifically express Foxp3, a key control gene in their development and function (Fontenot, 2003; Hori et al., 2003; Khattri, 2003, Sakaguchi, 2005). With CD25 and Foxp3 as specific molecular markers for detecting and manipulating naturally occurring Treg cells, there is now accumulating evidence that the Foxp3+CD25+CD4+ Treg cell population is actively engaged in the negative control of a variety of physiological and pathological immune responses and can be exploited for the prevention or treatment of autoimmune diseases, transplantation tolerance, negative control of aberrant immune responses (such as allergy and immunopathy) and enhancement of host defense (such as tumor immunity and microbial immunity (Sakaguchi, 2004; Sakaguchi, 2005)).

Natural CD25+CD4+ Treg cells have several significant immunological features which generate immunological tolerance to self or non-self antigens. Amongst these features is their ability to recognize normal self antigens tumor-associated antigens (Nishikawa, 2004; Sakaguchi, 2005). When stimulated by their respective antigens, they can suppress autoimmunity, hamper tumor immunity and suppress graft rejection. A deficiency or functional alteration of costimulatory or accessory molecules on T cells or a cytokine (such as IL-2) can indeed break self-tolerance and cause autoimmune disease. This means that any genetic abnormality or environmental insult could be a cause of or predisposing factor for autoimmune disease, if it reduces the number or affects the function of natural CD25+CD4+ Treg cells and self-reactive T cells towards the dominance of the latter (Ginsberg-Fellner, et al., 1985; Sullivan et al., 2002; Sakaguchi, 2004; Sakaguchi, 2005). In a therapeutic context, there is accumulating evidence with animal models that natural CD25+CD4+ T cell populations expanded in vivo or in vitro can be used in prevention and treatment of ongoing autoimmune responses by suppressing the population expansion and function of effector T cells (Tang et al., 2004; Tarbell et al., 2004; Sakaguchi, 2005).

That many tumor-associated antigens are recognized by autologous T cells in cancer patients that are antigenically normal self-constitutes indicates that natural CD25+CD4+ Treg cells engage in the maintenance of self-tolerance and may concomitantly impede immunosurveillance against autologous tumor cells (Boon, 1994; Dunn, 2004; Sakaguchi, 2005). A crucial experiment for addressing the function of the Treg cells in natural self-tolerance is to determine whether their removal from the normal immune system can break self-tolerance, resulting in auto-immune disease. Attempts

have been made since the mid-1980's to resolve this issue and to identify the purported Treg cells by expression of particular cell surface molecules, such as CD5, CD4+CD8+, mature thymocytes in normal naïve mice (Sakaguchi, 2005). According to Furuichi et al., 2005, Treg cells suppress priming and/or expansion of antigen-specific CD8+ cells during DNA immunization and the pek CD8+ population is enhanced by depleting this cell population. The elimination of Treg cells may or their inhibition may be important in immunotherapeutic strategies to control HBV infection by inducing virus-specific cytotoxic T lymphocyte responses in chronically infected patients.

A significant percentage of melanoma-reactive TIL contain both CD8-positive and CD4-positive T cells. These observations led Robbins et al. (2002) to identify tumor-reactive CD4-positive cells in populations of TIL that are associated with in vivo antitumor responses. In previous studies, T cells generated in a mixed lymphocyte tumor culture appeared to recognize a shared Ag in the context of the HLA-DR 15 restricted element. Previous extensive analysis of the specificity of class-I restricted T cells from a patient with melanoma revealed that these T cells recognized a mutated, as well as several non-mutated tumor Ags. Additional studies that were conducted on TIL from the same patient indicated they contained CD4-positive T cells that recognized the autologous tumor that had been induced to express HLA class II molecules. Additional clones were found by Robbins et al. (2002) to recognize an epitope of gp 100 in the context of the same HLA-DR restriction element. These observations provide an impetus to develop strategies directed toward generating HLA class II-restricted tumor-restricted tumor reactive T cells.

According to Alexandrescu et al., (2005) macrophage activation, as measured by neopterin levels, may correlate with the survival of patients undergoing biochemotherapy, while the generation of nitric oxide, acting synergistically with chemotherapy in producing tumor cell killing, may be reflected in the overall response rate seen with the biochemotherapy combinations.

Over-expression of macrophage inhibitory factor is thought to be the primary cause of gastritis and peptic ulcer disease as associated with *H. pylori* and gastric carcinoma etiopathogenesis (Mathews and Butler, 2005). The next section discusses the potential for *H. pylori* disease to serve as a model for global clinical practice measures and approaches to the management of melanoma. Like malignant melanoma, gastric cancer incidence is increasing and the survival of esophageal, gastrointestinal junction and gastric cancers is poor given that they frequently present with locally advanced or metastatic disease (Varadhachary and Ajani, 2005). In both diseases there are no definitive clinical treatment guidelines, and treatment decisions are based on heuristic clinical practice. The advent of biomarker testing, currently under development, may provide additional indicators of treatment to augment that practice.

HELICOBACTER PYLORI: AN EXAMPLE OF IMMUNE REPONSE AND DRUG RESISTANCE

Immune Response to *H. pylori* Infections

H. pylori gastritis is associated with peptic ulcer diseases and gastric cancer (Parsonnet et al., 1991; IARC Working Group, 1994; Pignatelli et al., 2005). There is a remote possibility that elimination of the *H. pylori* infection may have adverse health

implications (e.g. antibiotic resistance), and therefore "simple" risk stratification and targeted chemoprevention is required (Malfertheriner et al., 2005). The effect of prophylactic *H. pylori* eradication on gastric cancer incidence in humans remains unknown. Results from randomized trials are eagerly awaited, but availability of strong conclusive results may take several years (Malfertheriner et al., 2005). A growing number of studies show considerable variation in risk for gastric cancer development, depending on *H. pylori* strain type and the genetic predisposition of the host.

Gastric epithelial dysplasia is the precancerous lesion of gastric cancer. However the mechanism that dysplasia evolves to malignancy is not clear. The helicobacter-specific in vitro co-culture system was established and used to study the role of CD4+CD25+ regulatory T cells (Treg) in gastritis development in mice with *H. pylori* infection (Raghavan et al., 2004). Effects of therapeutic immunization against *H. pylori* infection on the Treg function were studied to better understand the mechanisms leading to post-immunization in the mice. The suppressive efficacy of Treg isolated from the differently treated mice correlated closely with production of IL5 by the Treg and suppression of interferon-gamma and IL-2 production by the CD25-effector cells. The investigators demonstrated that in *H. pylori* induced chronic infection, antigen-specific Treg with differential efficacy in suppressing *H. pylori* pro-inflammatory T effector cells.

Regulatory T cells protect the host from autoimmune disease by suppressing self-reactive immune cells. As such Treg may also block antitumor immune responses. Recent observations by Linehan and Goedgebuurne (2005) and others showed that the prevalence of Treg is increased in cancer patients, particularly in the tumor environment. Studies in their mouse pancreas model suggest that the tumor actively promotes the

accrual of Treg through several mechanisms involving activation of naturally occurring Treg as well as conversion of non-Treg onto Treg. These studies focus on further defining these mechanisms with the ultimate goal of designing strategies that block Treg-mediated suppression in cancer patients.

The *H. pylori* immuno-dominant protein, CagA, is associated with severe gastritis and carcinoma (Brandt et al., 2005). Brandt et al. (2005) found that IL-8 release induced by CagA is a multifunctional protein capable of effecting both actin remodeling and potentiation of chemokine release. Cho et al., (2003) found that the apoptosis-inducing properties of vacuolating cytotoxin (VacA) exert their effects in both Kato III and AGS cells, regardless of the p53 status and suggest that VacA mediate the development of gastric diseases through cell cycle arrest in the G1 phase with activation of the caspase-3. Cytokines (IL-6 in particular) secreted by TLR-activated DCs render naïve T cells resistant suppression, thus enabling them to respond effectively to invading microbes (Pasare, 2003; Sakaguchi, 2005). Chen et al. (2005) detected abnormal cell kinetics correlated to *H. pylori* and CagA+ strain infection and reported that *H. pylori* extract initiates apoptosis in BGC-823 cells through activating tyrosine kinase caspase-1, -3, and DFF. According to Choi et al. (2003), *H. pylori*-induced activation, especially by the cagA+ strain may play a protective role against gastric epithelial cell apoptosis partially through maintenance of bcl-2 family gene expression.

Assuming that natural CD25+CD4+ Treg cells are key in prevention of some diseases in humans, as best exemplified by IPEX, this could be attributed in part to insufficient adaptive population expansion or activation of natural Treg cells because of less frequent opportunities for microbial infections in hygienic environments. For

example, *H. pylori* is known to occur in over 50% of the world's population (Mathews and Butler, 2005) with the highest incidence recorded in industrially underdeveloped areas, including Asia (70-80%) and Africa (70-90%) and a waning occurrence in developed areas such as North America (30-40%), South America (80-90%), and Europe (30-70%). Prevalence rates vary between populations and between groups within the same population (Marshall and Warren, 1984; Peek et al. 1995Covacci et al., 1999; Cover and Blaser, 1999; El-Omar et al., 2000; Kauser, Sierra, et al., 2004; Sakaguchi, 2005). Gastric cancer is the leading cause of death from cancer in Costa Rica and while the incidence rates are declining (Parkin 1999; Sierra, 2002; Kauser, Sierra et al., 2004), they are the highest in Latin America and internationally they are second only to those observed in Japan (Parkin 1999; Sierra, 2002).

Mouse models of *H. pylori* infection have improved the ability for scientists to study this organism and can be used to investigate the host mucosal response to infection, particularly early events post-inoculation. Previous studies have shown that *H. pylori* infection leads to an increased production of reactive oxygen species within the gastric mucosa which are thought to be a major role in the mediation of associated disease. Mathews and Butler (2005) propose that the severity of inflammation and damage associated with *H. pylori* infection is dependent on the ability of mucosal cells to counteract the increased load of oxidative species.

Results from a study by Sierra et al., (1993, 2002) analyzed blood samples from 276 children and young adults collected from the same areas in Costa Rica for serum antibodies against *H. pylori* and found IgG and IgA antibodies to be very high. According to Sierra et al. (1993, 2002, 2004), chronic inflammation processes such as

infection with *H. pylori* linked to gastric cancer may well be due to the generation of nitric oxide, nitrite, and nitrite by macrophages and other cell types in inflamed tissues (Sierra et al, 1993, Occchialini, Sierra et al., 2001; Sierra, 2002).

Pignatelli et al., (2001) found several histological targets involved in the DNA damage, mucosal iNOS expression, and protein damage in the inflammatory cells. The investigators studied antral biopsies obtained from 34 patients with chronic atrophic gastritis and peptic ulcer disease before and after bacterial eradication. The expression of inducible nitric oxide synthase (iNOS) and level of nitrotyrosine and 8-hydroy-2'-deoxyguanosine were assessed immunohistochemically as markers of nitric oxide production and of damage to proteins. The results yielded that gastric mucosal iNOS expression, protein, and DNA damage occur in subjects with *H. pylori* infection and peptic ulcers. These compartments are also involved in adenocarcinoma, lymphoid hyperplasia, and lymphoma. The results depicted early bacterial eradication in the early stages of gastritis as seen in a decrease in oxidative stress. In contrast, in subjects with moderate-severe gastritis, *H. pylori* resulted in iNOS reduction without significant reduction in protein and DNA damage. The investigators concluded that in patients with moderate-severe gastritis, bacterial eradication alone would not be efficient in reducing protein and DNA damage. As with the treatment of malignant melanoma, careful scrutiny is need when devising a treatment plan. Biomarkers that augment treatment decision can help guide treatment decisions however the in vitro tests must be accurate. This involves a great number of clinical trials and careful study. For example, Sierra and colleagues (2003) have found that serum pepsinogen (PG) concentrations reflect the morphological and functional status of the gastric mucosa. The study was carried out in order to

determine the appropriate cut-off point of PG for identifying gastric cancer in a high risk population in Costa Rica. The study population was comprised of 338 subjects without gastric cancer and 20 gastric cancer patients. Serum pepsinogen concentrations were assayed. The PG level and PG ratio were significantly lower in patients with gastric cancer than in control subjects. The investigators found the best cut-off point to be concentrations < 60 mg/L and a ratio of PG I to PG II < 2.5 in screening for gastric cancer. Sensitivity was 90% and specificity was 64%. Low serum PGI concentrations and low PGI/II ratio are predictive of gastric cancer in this population. The investigators indicate that PG screening is simple and inexpensive however the investigators acknowledge that the real benefits of this test need to be defined by determining the impact on gastric cancer mortality rates.

Decreased Mortality and Drug Resistance in Drug Treatment Determination on Global Scale

While the ultimate reason to consider utilization of a novel diagnostic test is to improve mortality, other parameters must be taken into consideration, for example, drug resistance which develops over protracted time as a result of over-treating.

Magalhães et al. (2002) detected drug resistance to standard *H. pylori* treatment (metronidazole and clarithromycin) via gastric specimen isolates cultured under microaerobiosis method. The investigators examined isolates from 203 pretreatment patients with gastritis only (38.42%), with peptic ulcer (45.32%), and with gastric cancer (16.26%). DNA was extracted by a standard phenol-chlorform method. Both cagA and cacA genotypes were detected by PCR. Metronidazole MIC's showed a continuous distribution across the drug concentrations employed. Resistance may be linked with

frequent use of metronidozole, for example 70% in Brazil (Mégraund, 1998; Magalhães et al., 2002) in treatment of parasitic infections and *H. pylori.*

To complicate the understanding of drug resistance, it has been postulated that the progression of helicobacter-induced gastritis and gastric atrophy mediated type 1 T-helper cells may be modulated by concurrent parasitic infection. Fox et al. (2000) found that with concurrent helminth infection, helicobacter-associated gastric atrophy was reduced considerably in mice despite chronic inflammation and high helicobacter colonization. This correlated with a substantial reduction in mRNA for cytokines and chemokines associated with a gastric inflammatory response type 1 T-helper cells. Fox and others conclude that concurrent enteric helminth infection can attenuate gastric atrophy, the pre-malignant lesion.

Moreels and Pelkmans (2005) found helminthic parasites protect against T helper type 1 cell-mediated gastrointestinal inflammation like Chron's disease. Both TH-2 cells and regulatory T cells may be involved in this immunomodulatory mechanism. In their study, Moreels and Pelkman review the experimental and clinical studies in favor of the hygiene hypothesis, opening perspectives on new therapies for inflammatory bowel disease. Whary et al. (2005) found that Colombians living in coastal Tumaco have a lower incidence of *H. pylori*-associated gastric cancer compared with residents of Pasto in the high Andes. Considering that the risk for *H. pylori* disease seems affected by features of bacterial virulence and host polymorphisms and possibly concurrent helminthiasis, Whary et al. suggest that intestinal helminthiasis in children promotes Th2-polarizing response to *H. pylori* that may decrease gastric cancer risk in the individuals progression of gastric atrophy, dysplasia, and cancer. Studies are underway (Zeledón

(1999), Zeledón et al. (2001), Zeledón et al, (2002) and as of yet unpublished work by Zeledón, 2005) to analyze regions of the mitochondrial DNA of parasites in T. dimidiate parasite in Costa Rica to differentiate parasitic populations those that practice coprophagy and those that do not, a practice necessary to pass modified bacteria from one bug to another. The protection from parasites against T helper type 1 cell-mediated activity has become of interest in uncovering mechanisms of immunologic advance and the study of oncology where associations are beginning to be made.

Despite initial optimism that combination anti-*H. pylori* therapy ultimately eradicate gastric adenocarcinoma, recent investigations suggest its use should be targeted and tailored to a selected patient group considering the multifaceted role of *H. pylori* in disease and the disease heterogeneity of gastric adenocarcinoma (Wu et al., 2005). The clinical spectrum of *H. pylori* infection ranges from asymptomatic gastritis and peptic ulcer to gastric malignancies. The occurrence of one versus another is the result of differences in the magnitude of gastritis, and the current disease paradigm suggests gastric inflammation is common to all *H. pylori*-associated gastro-duodenal diseases. Therefore the host inflammatory responses to environmental triggers, rather than to bacteria or environmental factors, per se, would dictate the variable outcomes of *H. pylori* infection. Putative factors that are expected to play an important role in stimulating inflammatory pathways and modulating the cross-talk between host and environment are age at the time of infection, environmental cofactors, *H. pylori* virulence, and host genetics (Borody et al., 2002).

Elucidation of the intimate relationship between host-environment interaction and gastric inflammation, although currently a formidable task, is essential in the

development of new prevention and treatment strategies. Such knowledge according to Wu et al. (2005) might provide clues that allow more accurate prediction of variable outcomes of gastric inflammation and appropriate adjustment of treatment strategies, and might open up novel areas for studying gastric carcinogenesis. The evolving new technologies, such as in vitro micro-array, proteomic, and functional genomic analysis, promise to shed new light on the immense complexity of the presumed host-environment interactions and will reveal more useful markers for the diagnosis and prognosis of malignancies. Uncovering mechanisms of immunosurveillance in malignancies offers further development of diagnostics and therapeutics scientifically and ultimately for the physician treating the malignancy. The next section discusses immunosurveillance in malignant melanoma.

IMMUNE SURVEILLANCE IN MALIGNANT MELANOMA

Histopathologic Identification

The concept of immune surveillance was introduced by Burnet (Burnet, 1970). Burnet hypothesized that the development of T-lymphocyte-mediated immunity during evolution was specific for elimination of transformed cells. Donawho and Kripke (1992) examined mice exposed to UV light as it leads to the generation of suppressor T lymphocytes. The investigators discovered pathways to prevention of the immunologic destruction of these highly immunogenic tumor cells. UV radiation also influences the susceptibility of mice to systemic tumor challenge as shown by the fact that UV-light induced tumors form more pulmonary metastases in UV-irradiated mice than in normal syngeneic recipients (Donawho and Kripke, 1992 and Fidler, 2004). These experiments

demonstrate that even when tumors are highly antigenic, the primary host may be unable to eliminate metastases by immunologic means. Therefore, both the intrinsic antigenicity of the tumor and the ability of the primary host to respond immunologically to these antigens are important factors in the immunotherapy of metastases.

More recently Shankaran et al. (2001) found that immunodeficient mice were significantly more susceptible to formation of chemically induced tumors and spontaneous tumors than immunocompromised mice. Patients with immunodeficiencies show an increased incidence to virally induced tumors; however they also showed a higher incidence of tumors with no apparent viral etiology such as malignant melanoma, lung cancer, pancreatic cancer, colon cancer, and kidney cancer. In addition it was found that the presence of lymphocytes in such tumors is positively correlated to increased patient survival, especially in malignant melanoma. Studies in both mouse and human models by Dunn et al (2002) suggest that continuous pressure of the immune system in an immunocompetent host determines to a great degree if and how tumors evolve (immunoediting).

Pathways of Drug Resistance

Drug resistance, either at the time of the initial treatment or during relapse after remission, occurs in all but the few cancer types that are curable with chemotherapy. Drug resistance is a complex process involving multiple mechanisms that may emerge in parallel or in series. Many sources and pathways of drug resistance have been described in the literature in the past three decades (O'Brien and Cordon-Cardo, 1991; Weisenthal et al., 1991; Chu and DeVita, 2001; Berardi et al., 2004; Goedegebuure et al., 2004; Fidler, 2004). More than 70% of solid tumors fail to respond to chemotherapy

(Weisenthal et al., 1991). Ovarian cancer is one of the few malignancies where chemotherapy can lead to improved patient survival. However, the majority of these patients eventually relapse with disease resistant to further therapy.

Current models postulate that drug resistance is either intrinsic, existing prior to therapy, or is acquired as a result of selective and inductive pressures exerted on the cancer cell genome by the toxic effects of chemotherapy. Several groups have identified this expression with in vitro drug resistance assays upon initial diagnosis with cancer (Gazdar, 1990; Von Hoff, 1990; Von Hoff, 1991; Weisenthal et al., 1991; Wilbur, 1992; Shaw, 1993, Maenpaa, 1995; 1996; Cortazar, 1997; Kurbacher, 1998; Xu, 1999; Loizzi, 2003; Schrag et al., 2005).

It now appears that the capacity of certain cancers to resist the cytotoxic effects of cancer chemotherapy may be connected to either abnormalities in the genetic machinery of cancer cells or to alterations in the critical pathways of cell-cycle checkpoint control and apoptosis rather than to the specific mechanisms of resistance unique to each agent. Programmed cell death, apoptosis, is a distinct genetic and biochemical pathway essential to metazoans (Danial and Korsmeyer, 2004). An intact death pathway is required for successful embryonic development and the maintenance of normal tissue homeostasis. Apoptosis has proven to be tightly interwoven with other essential cell pathways. The identification of critical control points in the cell death pathway has yielded fundamental insights for basic biology and provided rational targets for new therapeutics. Both during tumor promotion and tumor progression, additional genetic mutations occur in tumor cells. These result in the formation of subpopulations of tumor cells that may or may not

grow out. Through a process of natural selection, this leads to heterogeneity within a tumor.

The clonal and genetic diversity of tumors permits both adhesion (adhesion theory) and detachment from the same matrix. Some cells within the primary tumor have the correct genotype. This observation is underscored by the general failure to overcome the resistance unique to chemotherapy in the clinical setting with approaches that attack only the classic biochemical or molecular mechanisms of resistance, or both (Danial and Korsmeyer, 2004).

With the availability of both innate and acquired immunity it is curious why these systems fail in cancer patients. Extensive research has demonstrated the existence of multiple escape mechanisms permitting tumor cells to escape from elimination by the immune effector cells. Only those cells that do show a sufficiently distinct phenotype from normal cells are expected to induce an immune response (O'Connell et al., 1999; Goedegebuure et al., 2004). At the early stages of tumor development the cells may in fact not express tumor antigens that are recognizable by the T cells and/or B cells, or if they do, perhaps it is at levels not sufficient to cause a response. Thus they may escape from innate effector cells. After the many mutations, the tumor cells may become more distinct from normal cells (Hussein, et al., 2003; Goedegebuure et al., 2004).

Melanoma cells can undergo self-destruction via programmed cell death, i.e. apoptosis. In melanoma tumors, the molecular components of apoptosis include positive (apoptotic) and negative (anti-apoptotic) regulators. The former include p53, Bid, Noxa, PUMA, Bax, TNF, TRIAL, Fas/FasL, PITSLRE, Interferons and c-KIT/SCF. The latter include Bcl-2, Bcl-XL and others (Hussein et al., 2003). Some of these molecules are of

potential therapeutic use, such as 1) p53, which influences resistance to chemotherapy; 2) Mcl-1 and Bcl-XL, which can override apoptosis; and 3) TRIAL, which has selective fatal effects on tumor cells Apoptosis, physiologic programmed cell death is different than non-physiologic accidental cell death or necrosis. In some tumors, including lung cancer, pancreatic cancer, melanoma, and others, cells may express the apoptosis-inducing molecule, Fas ligand, on the cell surface that can bind to the Fas receptor (FasR) expressed on activated resting T cells, and thereby induce apoptosis in T cells. Apoptosis will be discussed next.

Apoptosis in Melanoma

DNA damage initiates damage response pathways, cell cycle arrest, and apoptosis. Apoptosis is a tightly regulated phenomenon ensuring that cells that accumulate irreversible DNA damage do not replicate (Berardi et al., 2004). Defects in apoptotic system may contribute in the pathogenesis and resistance of malignant melanoma cells to chemotherapy (Mustika et al., 2005). Apoptosis plays a pivotal role in the homeostasis of human cell proliferation. Defects in the apoptotic system are likely to play a role in oncogenesis.

Two distinct pathways leading to apoptosis by chemotherapeutics have been depicted. Both converge on the activation of downstream caspases (Nunez et al., 1998) and either is death receptor dependent (extrinsic pathway) or independent (intrinsic pathway). The extrinsic pathway is through the activation of death receptors such as fast or tumor necrosis factor-1, by their ligands (Green and Reed, 1998), whereas the intrinsic pathway is primarily linked to mitochondrial functions (Ashkenazi and Digit, 1998). The drug-target interaction acts as a stimulus to initiate a cascade of events directed at the cell

cycle and eventually resulting in physiologic cell death (Goedegebuure et al., 2004). Cell death arises as a direct consequence of the drug-receptor interaction.

These processes act in a concerted fashion and remain functionally linked through mechanisms not completely understood. The critical molecular mechanisms involved in facilitating the initial coupling of the stimulus to the final response of the cell are still under study. This pathway involves some type of sensor that detects a death-inducing signal, a signal transduction network, and execution machinery that facilitates the process of cell death. This entire process is exceedingly complex as it is highly dependent on the specific cell type under study, the specific anticancer agent being tested, and the cellular context and environment in which the drug-target interaction is being considered.

One of the most remarkable features of both radiation therapy and chemotherapy, when used to treat sensitive tumors, is that their cytotoxic effects may be greater in neoplastic cells than in normal host tissues, including the bone marrow and the GI tract. Normal tissues almost never develop resistance to chemotherapy. Doses that eradicate some sensitive tumors will not ablate the bone marrow or destroy the capacity of the GI mucosa to regenerate. There was very little basis for this therapeutic selectivity until just recently (El-Deiry, 2005). However, according to El-Deiry, this difference in cytotoxic action between normal and malignant cells appears to relate to mechanisms that allow normal renewing cell populations, such as bone marrow and GI precursor cells, to monitor and repair damaged DNA or destroy cells with an irreparable DNA, rather than allowing damaged cells to proceed through the cell cycle and potentially replicate their damaged DNA.

Because they express intact genetic machinery, normal cells seem to almost always recover from exposure to DNA-damaging anticancer agents (El-Deiry, 2005), except in the case of high-dose chemotherapy, as used in transplantation programs. In this particular setting, the high doses of chemotherapy are able to overwhelm these protective mechanisms or to destroy the DNA of exposed cells by direct necrosis (or both). Initially, sensitive cancer cells can be destroyed by effective chemotherapy but, if not, they develop resistance to further treatment, perhaps in part because of drug-induced mutations in their DNA. This resistance may be linked to the dysregulation of the same genetic and signaling pathways that control entry into the cell cycle and the process of programmed cell death. Inactivation of apoptotic mechanisms, which can occur in addition to biochemical mechanisms associated with resistance to specific drugs or drug classes, can be mediated by inactivation of the p53 tumor suppressor gene (also termed the death pathway gene) or by inappropriate expression of genes that suppress apoptosis, such as bcl-2 (El-Deiry, 2005). The core molecular machinery of apoptosis is evolutionarily conserved, and so one might predict that the downstream elements would represent common targets for activation during tumor progression. Current thinking holds that caspase activation is the rate-limiting step in apoptosis and that activation of the so-called execution phase correlates with irreversible commitment to death (McConkey 2005; El-Deiry, 2005).

Protein p53 in Melanoma

The abrogation of the function of the "gatekeeper of the genome", p53 is the most molecular alteration in solid human tumors (Gods et al., 2005). Physiologic apoptosis appears to be regulated by many genes, but promotion of cell death by cytostatic drugs is

usually targeted at specific genes. These genes include both tumor suppressor genes such as p53 and oncogenes such as myc, E2F, c-jun, and bcl-2 (Brown and Outers, 1999; Goedegebuure et al., 2004). According to Gods et al., (2005) regarding melanomas the involvement of p53 alterations is discussed controversially to date.

The arrest of the cell cycle activated by p53 is thought to be critical in preserving the integrity of the cellular genome in response to treatment with a cytotoxic agent. The p53 gene appears to be rapidly upregulated after DNA damage and induces growth arrest by blocking cell cycle progression from G1 to S phase to allow repair of the DNA. The p53 gene is a tumor suppressor protein and critical transcriptional activator that appears to cause causes both G1 and G2 arrest of the cell cycle when cells are exposed to DNA-damaging agents. Once activated, p53 is thought to induce the expression of multiple transcriptional targets, including members of the bcl-2 family. These factors appear to contribute to the changes in mitochondria physiology that favor the release of cytochrome c (Stressed et al, 2000; Mustafa et al., 2005). Once in the cytosolic, chromosome c initiates formation of an oligomeric apoptotic protease-activating factor (Apaf-1) apoptosome. The apoptosome recruits and activates caspase-9, which in turn activates caspase-3 and caspase-7, which then kill the cell (Green and Reed, 1998; Mustika et al., 2005). The basis for the cell's response in arresting growth, repairing DNA, or inducing apoptosis remains unknown.

Members of the bcl-2 family promote or inhibit cell death via direct or indirect effects on caspase activation. Early work demonstrated that follicular bcl is driven by a chromosomal translocation that mediates high-level expression of Bcl-2 in follicular B-cells (Pegoraro et al., 1984; El-Deiry, 2005). Subsequent work showed that androgen

independence in prostate cancer is associated with increased Bcl-2 levels (McDonnell et al., 1992; El-Deiry, 2005). However, in other common solid tumors the role of Bcl-2 in tumor progression and response to therapy is less clear. For example, the majority of early studies showed that expression of Bcl-2 correlated with primary breast carcinoma (Harpin et al., 1997; El-Deiry, 2005) although more recent work suggests that higher levels of BCL-2 may correlate with poor response to neoadjuvant therapy (Buchholtz et al., 2003; El-Deiry, 2005). There is some evidence that Bcl-2 levels increase in metastatic melanoma, but reports arguing against this conclusion can also be found in the literature (Bush and Li, 2003; El-Deiry, 2005).

It is postulated that the ability to evade apoptosis is the key mechanism for the rapid growth of cancer cells. Melanoma is well known for its extreme chemoresistance and is poorly understood at the molecular level. Mutations in p53 are associated with poor prognosis, de novo or acquired resistance, and relapse in a broad field of solid and hematologic malignancies. Aggressive cancers, such as metastatic melanoma, that do not respond to traditional chemotherapy regimes are often resistant owing to their lack of p53-dependent apoptosis. However, despite the aggressiveness of metastatic melanoma, p53 mutations are rarely observed (Satymoorthy, 2001) and only found in about 15-20% of melanoma biopsies (Dai et al., 2004).

The initial studies showing that loss of p53 function associated with resistance to radiation therapy as well as chemotherapy came from in vivo model systems using p53 knockout mice (Lowe 1993a, 1993b, and 1994). Subsequent studies have confirmed malignant cell lines and tumors expressing mutant or deleted p53 are chemoresistant to a wide range of anticancer agents (Keurbitz et al., 1994). The p53 protein eliminates many

potential cancers by condemning damaged cells to death or quarantining them for repair (Tekautz, 2005). But the activity of p53 relies on intact active conformation which can be lost following mutation of a single nucleotide. Most apoptotic pathways involve a sensor that detects a death-inducing signal, a signal-transduction network and execution machinery that actively carries out the process of cell death.

p53 is the most frequently mutated tumor suppressor gene in human tumor samples (Dai et al., 2004) estimated to occur in at least 50% of all human tumors, however, loss of p53 is not always associated with chemoreisistance (Hollstein et al., 1991). While DNA damage can introduce apoptosis through a central sensor, (p53), p53-independent pathways exist as well. The ability of p53 to promote apoptosis in response to mitogenic oncogenes appears to be critical for its tumor suppressor function. With thousands of such mutations identified in patients, how can a future cancer drug buttress this fragile protein structure and restore the cell's natural defense? New therapies are being developed that aim to restore p53 tumor suppression to cancer cells. However, success towards this goal is extremely difficult. Recently, defects at other levels of the apoptotic network in melanoma have been discovered, adding further dimensions of complexity (Satymooorthy et al., 2000; 2001).

In the skin, excessive exposure to UV radiation induces apoptosis which presumably serves to eliminate heavily damaged cells and loss of p53 function leads to the survival of these damaged cells thereby initiating tumor development (Tekautz, 2005). While p53 was the first tumor suppressor gene to be linked to apoptosis, it seems there are a variety of signals which appear to be important in triggering apoptosis during tumor development. Extracellular triggers include radiation, growth or survival factor

depletion and loss of cell-matrix interactions and cell-cell adherence-based survival signals (Tekautz, 2005). Hence, loss of apoptotic function can impact tumor initiation, progression and metastasis.

Targeting survival and pro-apoptotic pathways simultaneously might be an efficient therapeutic strategy in melanoma. There are more than 100 transcription factors that have been identified (Goedegebuure et al., 2004) and their biologic roles are not fully understood. This is undoubtedly a complex issue that must take into account the extent of DNA damage, the stage of the cell cycle at which DNA damage occurs, the presence of other genetic abnormalities in ether the cell-cycle regulatory apparatus or the signaling machinery, or the specific cellular context.

Further worsening the problem of understanding the role of the many transcription factors and their role in apoptosis is the fact that several transcription factors activate downstream target genes, whereas others down-regulate expression in response to DNA damage. The target genes play an overall role in the outcome of the cell's fate. Research is beginning to uncover the possible cell cycle pathway mechanisms in melanoma that are involved in apoptosis which lead to chemotherapy resistance, despite a fully functional p53. Important in the discussion of cell death is Apaf-1 and its inverse correlation and the pathologic stage of melanoma.

Cell Death Effector Apaf-1

Recently it was found that Apaf-1, a downstream target of p53, is inactivated in metastatic melanoma. It has been shown that melanoma cells of different progression stages may avoid apoptosis by inactivating Apaf-1, thus disabling the p53 apoptotic program (Tekautz et al., 2005; El-Deiry, 2005). Apoptotic protease-activating factor-1

(Apaf-1) is a mitochondria-associated protein and cell death effector that acts with cytochrome c and binds procaspase-9 to activate the enzyme to mediate apoptosis. Caspase-9 and its cofactor Apaf-1 were found to be essential downstream components of p53 in Myc-induced apoptosis in a study by Soengas et al. (1999). Like p53 null cells, mouse embryo fibroblast cells deficient in Apaf-1 or caspase-9, and expressing c-Myc, were resistant to apoptotic stimuli that mimic conditions in developing tumors. Inactivation of Apaf-1 or capase-9 substituted for p53 loss in promoting the oncogenic transformation of Myc-expressing cells. These results imply a role for Apaf-1 and caspase-9 in controlling tumor development as suggested by Lowe et al. (2001) and Soengas (2001). Apaf-1-negative melanomas are reported to be chemoresistant and are unable to execute a typical apoptotic program in response to p53 activation. Restoring physiological levels of Apaf-1 through gene transfer or 5-aza-2′-deoxycytidine (5aza2dC) treatment was found to greatly enhance chemosensitivity and rescue the apoptotic defects associated with Apaf-1 loss. Pretreatment assessment of Apaf-1 status could improve the therapeutic management of patients with melanoma (Lowe et al., 2001; Soengas et al., 2001).

In 2001, Soengas et al., surveyed several tumor types for loss-of-function mutations in the Apaf-1 gene. They included melanoma in their analysis because of it has extreme drug resistance to apoptosis-inducing agents but retains wild-type p53. They analyzed a series of metastatic melanoma samples for their p53 and Apaf-1 status. A low rate of mutation was detected for p53 and in contrast, a markedly high rate of allelic loss was found for polymorphic markers encompassing the Apaf-1 locus. Moreover, they found that tumors with loss of heterozygocity expressed little Apaf-1 message by in situ

hybridization compared with tumors with no loss of heterozygosity. Only about one out of six primary melanomas analyzed showed reduced Apaf-1 expression. According to Dai et al., (2004) loss of heterozygosity of the Apaf-1 gene was found in 40% of metastatic melanoma. The authors concluded that loss of Apaf-1 is a relatively common event in melanoma and may be associated with progression of the disease.

In the same study, Soengas et al. also reported that that 10 out of 19 metastatic melanoma cell lines and 10 out of 24 melanoma specimens expressed low levels of Apaf-1. They found that ApaF-1 expression inversely correlated with the chemosensitivity of melanoma cell lines in vitro. Based on these observations, they proposed that Apaf-1 down-regulation contributes chemoresistance in malignant melanoma. The authors also suggest that Apaf-1 down-regulation may be a late event during tumor progressions (four out of five in situ melanomas were positive for Apaf-1).

Mustika et al. (2005) evaluated Apaf-1 protein expression by immunohistochemistry in 10 cases of human nevi, 11 melanomas in situ, 26 primary melanomas and 15 metastases. The authors observed significant decreases in Apaf-1 expression when comparing nevi and melanomas (chi square=33.719; $P< 0.0001$). Moreover, primary melanomas with greater tumor thickness showed lesser expression of Apaf-1 (chi square=16.182; $P<0.003$). Intriguingly, the authors were unable detect Apaf-1 expression in lesions of metastatic melanomas. These data demonstrated there is an inverse correlation between Apaf-1 expression and pathologic stage of melanoma. This suggests that the decreased expression of Apaf-1 seen in correlation with melanoma progression renders melanoma more resistant to chemotherapy.

To determine if loss of Apaf-1 expression is involved in melanoma progression, Dais et al (2004) employed tissue micro array technology and examined Afaf-1 expression in 70 human primary malignant melanoma biopsies by immunohistochemistry. The data showed that Apaf-1 expression significantly reduced in melanoma cells compared with normal nevi (chi (2) =6.02, P=0.014). The results also revealed that loss of Apaf-1 was not associated with the tumor thickness, ulceration or subtype, patient's gender, age and five-year survival. In addition the in vitro apoptosis assay used in the study revealed that over-expression of Apaf-1 can sensitize melanoma cells to anticancer drug treatment thus lending itself as a possible therapeutic target.

In their 2005 article in Cell Death and Differentiation, "Apaf-1 expression in malignant melanoma," Soengas et al., summarize their work to date comparing and contrasting their findings with that of others. Peltenburg, et al. (2005) and Allen, et al. (2005) imply that Afap-1 down-regulation is not the only determinant of chemoresistance in melanoma. Soengas et al. (2001) agree as highlighted in their findings of a drug-resistant line (SK-Mel-173) that retained high Apaf-1 expression. In the same study, the authors frequently detected allelic loss, however mutations of Apaf-1 gene were not detected in eight of the cell lines tested which suggests that Apaf-1 is not a classical tumor suppressor and that other mechanisms must exist which abolish Apaf-1 activity. Nonetheless, Soengas et al., maintain that there is evidence to suggest that Apaf-1 levels can affect apoptosis in melanoma cells citing six recent studies that analyzed over 400 pigmented lesions to support the down-regulation of Apaf-1 during melanoma progression (Furukawa et al., 2002; Baldi et al., 2004: Dai et al., 2004; Fujimoto et al., 2004; Qin et al., 2004; Zanon et al., 2004; Mustika et al., 2005).

To conclude, malignant melanoma develops through the malignant transformation of melanocytes and is famous for its tendency to metastasize early in the course of the disease. This tumor is extremely resistant to induction of apoptosis by chemotherapy suggesting that in this tumor, the apoptosis machinery is defective. Soengas and colleagues, by in vivo and in vitro experiments, recently showed that metastatic melanomas often loose Apaf-1. Loss of Apaf-1 confers chemoresistant properties and inability to execute a typical apoptotic program in response to p53 activation (Soengas et al., 2001). It remains elusive however whether loss of Apaf-1 is also observed in vivo or associated with progression to disease. Accordingly, in the study by Mustika et al. (2005), the authors attempt to investigate the expression of the Apaf-1 protein in nevi, melanoma in situ, primary invasive melanomas as well as metastatic human melanomas. The author's data clearly demonstrate a decline of Apaf-1 protein levels with the progression of disease. The authors found a significant decrease in Apaf-1 expression when comparing nevi to primary and metastatic melanomas. Apaf-1 expression in melanoma in situ was as strong as nevi, indicating that the decrease in expression was not an early event but a late event in tumor progression. In fact, metastatic melanomas had no Apaf-1 expression. Furthermore the author's results also revealed that loss of Apaf-1 was significantly associated with the tumor thickness. These findings are in agreement with Baldi et al. (2004), although in the Mustika study, 10 of 26 melanoma cases were ALM, the prevalent type in Japan. The authors suggest that the decreased expression of Apaf-1 seen in correlation with melanoma progression renders melanoma more chemoresistant. Therefore, there is a possibility that restoring physiologic levels of Apaf-

1 through gene transfer or a methylation inhibitor would enhance chemosensitivity and thus have therapeutic benefit to melanoma.

Kim et al. (2004) pursued the hypothesis that apoptosis in vivo would distinguish metastatic cells from non-metastatic cells and developed a novel method for observation of apoptotic induction in living cells as they observed the translocation of proteins to the mitochondria during apoptosis in isolated lung preparations in rats. The authors found many more of the translocations of proteins in the arrested, non-metastatic tumor cells than of the metastatic melanoma cells (MMCs). TUNEL staining confirmed enhanced apoptosis by non-metastatic tumor cells after injection in vivo. MMCs or embryo fibroblasts were better able to negotiate the barrier of survival in the circulation after pulmonary arrest than non-metastatic cells confirming the hypothesis that susceptibility to apoptosis after arrest in the pulmonary vasculature distinguishes metastatic from non-metastatic cells and introducing a new assay for in vivo induction of apoptosis (Kim et al., 2004). Much remains to be done to further understand cell death pathways. As the absence of functioning pathways is related to tumor development and the ineffectiveness of drugs, extending our knowledge in this area is crucial. Next brief mention is given to the development of metastasis and as it pertains to the understanding of tumor resistance and as an example of complexity facing the pathologist in histology interpretation.

Development of Metastasis

Only few cells in the primary tumor give rise to metastasis (Folkman, 1999; Fidler, 2004). This is in part due to the elimination of the disseminating tumor cell which fails to complete a step in the metastatic process. Most data agree that neoplasms are biologically heterogeneous, that the process of metastasis is selective or that only specific

and unique cells possess the appropriate properties that enable them to survive the potentially destructive journey form the primary tumor site to the metastatic site(s).

The formation of tumor metastases is characterized by detachment of tumor cells from the primary tumor which infiltrate into the bloodstream or lymphatics. The reciprocal process occurs at the other locations in the body. Both processes are characterized by changes in the extra-cellular make-up and interaction with tumor cells (Robbins et al., 2002; Ricci et al., 2004). Like tumor promotion, tumor progression may be also dependent on infiltration. The difference between the growth of tumor at an earlier stage and that of later stages may be that at later stages more tumor cells have acquired a stimulatory paracrine loop of growth factors as a result of continuous transformations in tumor cells and the selection for survival. For example, over-expression of extracellular matrix receptors in breast, lung, bladder cancer and melanomas are often associated with poor survival and enhanced metastases (Ricci et al., 2004).

Like many neoplasms, melanomas can have a multi-cellular origin. The zonal differences include biologic characteristics such as growth rates, sensitivity to cytotoxic drugs, antigenicity, and pigmentation (Folkman, 1999; Fidler, 2004). Histopathologically the malignant or benign nature of a tumor may be difficult to determine with confidence as often it is more accurate if sections from all parts of the tumor are examined.

Neoplastic cells are genetically unstable and as such are heterogenous for a large number of biologic properties that include invasion and metastasis. The process of metastasis consists of sequential and selective steps that are highly selective. To produce a metastasis, tumor cells must complete all steps, including proliferation, induction of

angiogenesis, motility, and invasion, entrance into the circulation, arrest in a distant vascular bed, intravasation and extravasation into visceral parenchyma, proliferation, and induction of neovasculature. At all steps, the tumor cells must evade destruction by host-specific and nonspecific defense mechanisms.

Distinguishing benign nevi from malignant melanomas histologically can be challenging and is one of the greatest sources of debate amongst pathologists (Barnhill et al., 1995-2002; Li et al., 2003; Dolled-Filhart Rimm, 2002). Li et al. (2003) and Barnhill et al. (1995) evaluated the combined diagnostic abilities of multiple cytometric markers in separating various types of nevi from melanomas (Li et al., 2000, 2002, and 2003). Li and Barnhill discovered benign and malignant melanocytic lesions have differing characteristic features regarding nuclear DNA content, nuclear morphology, transcriptional activity of nuclear organizer regions, and cell proliferating activity. The study by Botonni et al. (2003) sought to combine the cytometric techniques to produce an enhanced method of separating benign nevi from melanomas. The investigators found diagnostic effectiveness can be improved by co-evaluating multiple cytometric features of tumor cells in melanocytic lesions. This may be of particular significance in the differentiation of some melanocytic lesions whose biologic behavior cannot be confidently predicted by their histologic features (Li et al., 2003). Other cytometric techniques have been developed recently and show some promise in this regard. These include DNA microdensitometry and karyometry (Li et al., 2003), quantification of argyrophilic staining of nuclear organizer regions (agNORs) (Crocker et al., 1987-2003) and immunoreactivity of cell proliferation markers such as MIBI-Ki67. Distinguishing benign from the malignant tumor continues as a source of study and challenge.

Multi-drug resistance as briefly described in the next section exerts challenge in diagnostic and therapeutics of malignant melanoma and overshadows attempts to unravel the intricacies of tumor behavior. Studies of in vitro testing of multi-drug resistance of bacteria lead to the development of in vitro testing for tumor cells.

MDR-Multi-Drug Resistance testing for bacteria and tumor cells

The term multi-drug resistance (MDR) describes the observation that tumor cell lines can become cross-resistant to several structurally unrelated chemotherapeutic agents after exposure to a single cytotoxic drug (Biedler and Riehm, 1970; Covelli, 1999). Among other things, this suggests that some caution should be used in the choice of a therapeutic approach due to the possibility of cross resistance. The seminal work of Biedler and Riehm (1970) laid the foundations for subsequent developments regarding MDR and oncology (Kerbel, 1994; Desoize, et al., 1998, 2000).

Hematologic malignancies, such as acute myeloid leukemia (AML), multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL) are characterized by initial sensitivity to cytotoxic drugs in the majority of the patients with later development of chemotherapy-resistant disease upon relapse (Desoize, et al., 2000). Goldie and Coldman (1983) hypothesized that a small number of resistant cells are present at diagnosis or may develop during treatment through spontaneous mutations that later overgrow the sensitive cell population under the selective pressure of cytotoxic drugs.

Studies in solid tumors such as those that result from metastatic melanoma are technically different than studies in hematologic neoplasms because solid tumors most commonly are present as three-dimensional aggregates of cohesive cells while hematologic neoplasms are almost exclusively discohesive (Kobayashi et al., 1993).

Studies have shown that in vitro drug activity correlated with in vivo drug activity when tumors were tested in vitro as three dimensional clusters, but not when they were tested in two dimensional mono-layers (Desoize, et al., 2000).

For many years, clinical trial design that sought to examine therapy for malignancies had been dominated by the use of alternating cycles of combination chemotherapy. The basis of this study design came from the translation of preclinical experimental data into a model for clinical treatment (Luria and Delbruck, 1943). In 1943, Luria and Delbruck observed that the bacteria, Escherichia coli, developed resistance not by surviving exposure but by expanding clones of bacteria that had spontaneously mutated to a type inherently resistant to phage infection. This was a seminal principle in bacterial genetics and laid the framework for the development of the notion of spontaneous resistance to cancer chemotherapy.

Goldie and Coldman Principle of Drug Resistance

In 1979 Goldie and Coldman applied this principle to the development of resistance to anticancer drugs by cancer cells without prior exposure to these drugs. They proposed that the nonrandom cytogenetic changes now known to be associated with most human cancers probably were tightly associated with the development of the capacity to resist the action of certain types of cancer drugs (Goldie and Coldman, 1979). The investigators developed a mathematical model that predicted that tumor cells mutate to drug resistance at a rate intrinsic to the genetic instability of a particular tumor.

The model predicted that mutations resulting in drug resistance would begin to occur in population sizes between 10^3 and 10^6 tumor cells (1000 to 1 million cells), much lower than the mass of cells considered to be clinically detectable (10^9, or 1 billion cells).

The probability that a given tumor will contain resistant clones when a patient's disease is newly diagnosed would be both a function of tumor size and the inherent mutation rate. If the mutation rate is as infrequent as 10^6, a tumor composed of 10^9 cells (a1-cm mass) would be predicted to have at least one drug-resistant clone; however, the absolute number of resistant cells in a tumor composed of 10^9 cells would be relatively small. Therefore, such tumors should initially respond to treatment with a partial or complete remission but would recur as the resistance clone expands to repopulate the tumor mass. Such a pattern is commonly seen in the clinical setting with the use of chemotherapy in many drug-responsive tumors.

The Goldie-Coldman hypothesis predicted that cellular drug resistance should be present even with small tumors and that the maximal chance for cure occurs when all effective drugs are given simultaneously (Norton and Day, 1991). Because this would involve at least a dozen chemotherapeutic drugs be given simultaneously, this approach has not been tested clinically in humans, as more than five cytotoxic drugs, at full doses, would not be possible. An alternate approach, using two programs of effective, non-cross resistant drug combinations in altering cycles has been used and evaluated since the 1980's.

In 1989, Day reanalyzed the Goldie-Coldman hypothesis to verify the basic tenants of the Goldie-Coldman model; however, they reportedly believed that the sequential use of combinations should outperform alternating cycles as no two combinations are likely to be strictly non-cross resistant or have equal cell-killing capacity – this was the symmetry condition assumed by Goldie and Coldman (Slamon, et al. 1989). Day's "worst drug rule" model referred to any strategy which administered

greater quantity or earlier doses of a treatment found to be the least effective of two or more available options (Tsujimoto, et al., 1985; Chu and De Vita, 2001). This had interesting implications which involved a very intuitive approach as follows: If treatments A and B are available and B is known to be better, a physician is likely to use B first. Cells that are resistant to the superior treatment, B, must be eliminated by the weaker program, A; however, because it is the weaker program, the physician cannot wait too long to use it or the overgrowth of the population resistant to B will place the clinician and patient in a situation that is difficult to overcome. The model predicts that if six cycles of A and B are planned, use of the weaker program A, first, offers a better outcome. There have been clinical examples in which sequential therapies have outperformed alternating cyclic use of the same programs if the dose intensity of the two regimens is carefully controlled.

This model and others (Gardner and Ferendes, 2003; Edelman et al., 2004) continue to serve as a construct for treatment planning; however, there is no model that can address or begin to resolve all the many issues facing the oncologist or the clinician and the patient. The now-familiar-two-week interval between cycles of the most effective drug combinations, using standard doses to accommodate the recovery of bone marrow is often used. This schedule neither accommodates successfully the rapid re-growth and establishing intervals often permits the tumor volume to return to pretreatment levels. No rigid schedule can accommodate all the variables assumed to be important for maximum effectiveness of combination therapy and the requirements of the patients in the practice of medical oncology. With advanced melanoma, the schedules are modified from the "standard" as a means to gain control of the tumor spread when it is found that the

standard treatment(s) have failed. This modification was observed in the treatments for the patients with advanced melanoma as treated by the oncologists in the Yale Cancer Center Melanoma Group as discussed further in the document.

The omission of a drug from a combination may allow overgrowth by a cell line sensitive to that drug alone and resistant to other drugs in combination. The arbitrary reduction in the dose of an effective drug to add other less effective drugs may dramatically reduce the dose of the most effective agent below the threshold of effectiveness and destroy the capacity of the combination to cure disease in a given patient (Goldie, 2001). When drugs must be discontinued or dosing reduced secondary to worsening of underlying illness or toxic side effects, the oncologist bears in mind the complications that arise in parallel with such discontinuation or reduction in dosing (Goldie, 2001). Many questions regarding interpretation of response to chemotherapeutic drugs still go unanswered. The general concerns outlined here regarding the development of cross-resistance to drug therapies are operational in the development of treatment plans for melanoma patients.

DETERMINANTS OF TREATMENT PLANNING

Treatment of Malignant Melanoma

The treating physician weighs potential benefits of chemotherapy treatment against possible worsening of an underlying systemic condition. Consideration of histology, stage, and other tumor-related variables, together with the patient's age and baseline health, contribute to treatment determination factoring in the realistic opportunity for curative treatment. Side effects inherent to chemotherapy and

immunotherapy are significant factors in treatment planning. Quality of life is in great measure where consideration and decision making and planning focus within the relationship between the treating physician and patient with advanced melanoma.

A decision to treat with curative intent demands a high degree of adherence to drug dosing and scheduling requirements, as specified in the standard or experimental regimen, and an acceptance of treatment-related toxicity. When cure is not a realistic expectation, a decision to treat must be based on an expectation for prolongation of the patient's life or an improvement in the quality of life. In these cases, treatment-related side effects may be minimized by dosage adjustments or treatment delays, but at the cost of antitumor efficacy. When the probability for benefit is low, chemotherapy is offered after consideration of drug efficacy, possible drug toxicity, economic consideration and patient choice. Quality of life is an issue quite salient in the treatment of advanced melanoma and is discussed further in the section "Effectiveness of Tests: Quality of Life."

History of Chemotherapy

Paul Erlich coined the word "chemotherapy". Erlich's use of in vivo rodent model systems to develop antibiotics for treatment of infectious diseases led George Clowes, at Roswell Park Memorial Institute in Buffalo, New York in the early 1900's to develop inbred rodent lines bearing transplanted tumors that could be used to screen potential anticancer drugs. This in vivo system provided the foundation for mass screening of novel compounds (Marchall, 1964; Chu and De Vita, 2001). Alkylating agents represent the first class of chemotherapeutic drugs to be used in the clinical setting (Hersh, 1968; Alexander, 1944; Chu and De Vita, 2001). These agents were a product of

the secret gas program of the United States in both world wars. The exposure of military seamen to mustard gas in World War II led to the observation that alkylating agents caused marrow and lymphoid hypoplasia.

These observations led to the direct application of such agents in humans with hematologic neoplasms, including the lymphocytic lymphomas and Hodgkin's disease. The first treatments were administered at the Yale Cancer Center in 1943. However, given the secret nature of the gas warfare program, this work was not published until 1946 (Marchall, 1964; De Vita, 1978). The demonstration of dramatic regressions in advanced lymphomas with chemotherapy generated much excitement. At approximately the same time, Sidney Farber reported his findings that folic acid had a significant proliferative affect on leukemic cell growth in children with lymphoblastic leukemia (Chu and De Vita, 2001). These observations resulted in the development of folic acid analogs or cancer drugs, which would inhibit folate metabolism and is marked as the inception of the era of cancer chemotherapy.

The cure of childhood leukemias and Hodgkin's disease with combination chemotherapy in the 1960's proved the much disputed point that a fraction of human cancers, even in their advanced stages, could be cured by drugs. This seminal work laid the foundation for the application of chemotherapy in the treatment of solid tumors (Tsujmoto et al, 1985; Chu and De Vita, 2001). The most disappointing aspect of the work with solid tumors was the failure to cure more patients once it was shown that cancer cells might be more sensitive to cytotoxic drugs than normal cells.

This is an especially relevant issue in the adjuvant setting where, because of low tumor burden, cancer cells were thought to be more sensitive to eradication by drug

therapy (De Vita 1971, 1991). Chemotherapy failure was, at first, thought to be due to variations in tumor growth characteristics. Failure was eventually determined to be caused by specific and permanent mechanisms of resistance to individual chemotherapeutic agents that were either acquired after exposure to cancer drugs or were already present as a consequence of intrinsic genetic mutations within the tumor (Goldie and Coldman, 1984). Emphasis in the design of early studies was on focusing and maximizing the interaction of the active component of chemotherapeutics with the cycling cancer cell. According to Young and De Vita (1970), research revealed that cancer cells did not divide faster than normal cells; rather, a larger fraction of the population was dividing.

Much of the early clinical work in cancer chemotherapy was based on the kinetic modeling of the drug therapy of the murine leukemia L120 cell line. The work with the L120 model (De Vita, 1971) was also the basis for the long-held dogma that rapidity of growth and frequency of cycling in responsive tumors determined sensitivity to chemotherapy. Thus, slowly growing tumors are not kinetically vulnerable, whereas faster-growing tumors are both responsive and curable. Largely because of their good response to treatment, leukemias and lymphomas were considered to be rapidly growing.

This observation led to the odd conclusion that solid tumors, such as lung cancer, colon cancer, and other 'resistant tumors' such as metastatic melanoma, were slow-growing, even though there was insufficient evidence for this. This discovery ran counter to another important clinical observation, that certain human cancers that display a spectrum of growth patterns from indolent to aggressive become significantly more treatable as well as potentially curable as the cell of origin becomes less differentiated

and the growth rate, as measured by thymidine-labeling index, increases (DeVita, 1971). When this same tumor transforms however, to a highly aggressive phenotype, paradoxically it often becomes almost totally incurable.

Response Rate as Therapeutic Measure

Adjuvant chemotherapy, systemic treatment after the primary tumor, is has been controlled by an alternative modality, such as surgery and radiation therapy, usually is based on response rates in separate groups of patients with advanced cancers of the same histologic type. Radiation therapy has been recommended for patients with head and neck melanomas as well as mucosal melanomas in the pelvic region. Retrospective analysis suggests that this will reduce the incidence of local recurrence (Urist and Soong, 2004). The determination of a population of patients as suitable for adjuvant treatment is based on available data about their average risk of recurrence after local treatment alone (Eklund et al., 2005).

According to Sun and Schucter in the textbook, Cancer Treatment Options in Oncology, the major indicator as to the effectiveness of a chemotherapy program, the complete remission rate, is lost in the adjuvant setting because the primary tumor has already been removed. In the clinic, the treatment selection for individual patients is based on response rates in the population of patients with advanced disease of the same histologic type. In adjuvant programs, micrometastases may occur. These consist of a mixture of tumor cells, some of which could have been expected to be sensitive to chemotherapy and others which could have been expected to be resistant to chemotherapy. The relapse-free survival in the adjuvant setting, therefore, measures time to regrowth for clinically detectable levels of cells unresponsive, partially responsive, or

very sensitive to chemotherapy, and is the equivalent of the duration of remission of a combined group of complete responders, partial responders, and nonresponders.

Combination Chemotherapies

In the early years of cancer chemotherapy, drug combinations were developed based on known biochemical actions of available anticancer drugs rather than on their clinical efficacy (Nathanson et al., 1969; De Vita and Schein, 1973). These combinations were not very effective. The era of effective combination chemotherapy began when an array of active drugs from different classes became available for use in combination for the treatment of leukemias and lymphomas. Combination chemotherapy has now been extended to the treatment of most solid tumors including metastatic melanoma. Combination chemotherapy using conventional cytotoxic agents accomplishes several important objectives not possible with single-agent treatment. First, it provides maximal cell kill within the range of toxicity tolerated by the host for each drug as long as dosing is not compromised. Second, it provides a broader range of interaction between drugs and tumor cells with different genetic abnormalities in a heterogeneous tumor population. Finally, it may prevent or slow the subsequent development of drug resistance.

Most standard chemotherapeutic programs were designed around the kinetics of recovery of the bone marrow in response to exposure to a cytotoxic agent. Bone marrow has a storage compartment that supplies mature cells to the peripheral blood for 8 to 10 days after the stem cell pool has been damaged by cytotoxic drugs. Events in the peripheral blood are usually a week behind events occurring in the bone marrow. Many patients are pretreated with colony-stimulating factors (CSFs) which help to accelerate bone marrow recovery. This helps with the leucopenia and thrombocytopenia which is

usually observed on the ninth or tenth day after initial dosing. The onset of recovery generally begins day 21 and generally is completed day 28. This sequence is often altered however, in patients with previous therapy by depletion of the stem cell pool, shortening of the time to the appearance of leucopenia and thrombocytopenia and prolongation of the recovery time.

The highest risk of infection or bleeding occurs with the granulocyte counts lower than 500/dL and platelet counts lower than 10,000/dL. Repeated dosing during the phase of early recovery of the marrow (days 6 to 21) may result in more severe toxicity in the second and subsequent treatment cycles in patients whose marrow is not the source of, or involved with, the tumor. These challenging factors are ongoing realities in the treatment of malignant melanoma.

Ideally drugs should be used at their optimal dose and schedule, with drug combinations given at consistent intervals. Because long intervals between cycles negatively affect dose intensity, the treatment-free interval between cycles should be the shortest possible time necessary for recovery of the most sensitive normal target tissue, which is usually the bone marrow. The side effect profile along with underlying systemic disease often preclude sustaining this ideal as dosing often must be reduced or stopped to avoid complications resulting from the therapy. Physicians who treat malignant melanoma face many obstacles in establishing ideal therapies for each patient. The desire to use drugs at optimal doses and on an optimal time schedule has to be weighed against patient tolerance of the drug and individual specific health problems beyond the cancer being treated. Another issue is the current ineffectiveness of the available therapies. The

following section discusses specific therapeutic agents currently available. Their limitations and survival outcomes from clinical trials will be discussed.

Current Chemotherapy and Immunotherapy in the treatment of Malignant Melanoma

Metastatic melanoma is a capricious neoplasm that is associated with spontaneous regressions. Responses may be temporally but not causally related to treatment. Standard treatment for patients with metastatic melanoma has not been defined however single-agent chemotherapy is the mainstay of treatment for the majority of patients with malignant melanoma (Danson and Lorigan, 2005). There are no generally accepted, clinically effective treatments for stage IV melanoma (Danson and Lorigan, 2005). The range of treatment options includes close observation, surgical resection of isolated metastasis, single-agent chemotherapy, combination-chemotherapy regimens, immunotherapy, biotherapy, and/or participation in clinical trials. When feasible, it is ideal that patients with metastatic melanoma be encouraged to participate in clinical trials. A number of patients among the cohort examined for this research were referred to clinical trials at diagnosis or when it appeared their chemotherapeutic or immunotherapeutic regimen was failing.

Various chemotherapeutic agents have demonstrated activity in metastatic melanoma but at consistently low rates, i.e., less than a 25% response rate for any single agent (Nestle 2002; Kadison and Morton, 2003; Richtig et al., 2004; Urist and Soong 2004). Chemotherapeutic agents that have shown activity as single-agent therapy include dacarbazine (DTIC), cisplatin (Platinol-AQ), carmustine (BCNU), paclitaxel (Taxol), docetaxel (Taxotere) and temozolomide (Temodar), an oral form of dacarbazine but with

greater central nervous system penetrance (Richtig et al,. 2004) . Other cytotoxic agents with single-agent response activity have been tried alone and in various combination regimens include fotemustine, vindesine, cyclophosphamide, vinblastine, vincristine, hydroxyurea, bleomycin and docetaxel (Crosby et al. 2000; Sun and Schuchter 2001).

Dacarbazine (DTIC, di-methyl triazeno imidazole carboxamide) is the most effective drug used in patients with distant metastatic disease in melanoma and is the reference agent for its treatment in phase I and II clinical trials (Crosby et al., 2000; Sun and Schuchter 2001; Moretti, 2001; Richtig et al., 2004; Urist and Soong, 2004). Response rates demonstrate a yield of 20% for skin and lymph node metastases but less than five percent for visceral or skeletal metastases. When used alone DTIC has partial response rates (greater than 50% reduction in tumor size for a duration of more than 4 weeks) of approximately 15-28%, complete responses in 3-5%, and long term remissions of less than two percent (Crosby et al., 2000; Kadison and Morton, 2003). With concomitant administration of anti-emetics, DTIC is fairly well-tolerated.

Crosby et al. (2000) conducted a review of 944 abstracts of research results of Phase I and II randomized controlled trials drawn from various databases (Medline, Embase, Cochrane Trials Register, Database for Reviews of Effectiveness, and Science Citation Index). They reviewed the studies to assess the benefits from use of any systemic therapy compared with supportive care/placebo to establish a 'gold standard' therapy against which other agent(s) treatment(s) could be compared in future studies (Crosby et al., 2000). The review found "no evidence to support a single, tried and tested gold standard therapy against which other treatments can be compared" (Crosby et al., 2000). Although no randomized control trials were found comparing DTIC with best

supportive care/placebo, DTIC has been used in the control arm in more than 20 prospective randomized controlled trials (Crosby et al., 2000).

The combination of dacarbazine with a vinca alkaloid and an alkylating agent (cisplatin or lomustine) yields a response rate of 30% to 40%. These agents have shown an effect in metastatic disease with RR between 2.5% and 47% (Richtig et al., 2004) but so far, no therapy has been proven superior concerning survival of patients. It remains a matter of debate whether the combination of several cytotoxic agents may increase RR or survival, but combined chemotherapy is definitely more toxic (Richtig et al., 2004).

The addition of tamoxifen, an estrogen receptor blocking agent widely used in the treatment of breast cancer, has also been tested, usually in conjunction with cytotoxic agents. The question is whether tamoxifen will modify disease response to the combination drugs (Crosby et al., 2000). Several clinical trials claim no evidence that the addition of tamoxifen improves the response rate for malignant melanoma tumors and its toxicity was found to be substantial (McClay et al., 2001). The results of trials with some of these agents (platinum, vinca alkaloids, nitrosureas and taxanes) claim higher response rates but it remains unclear whether they offer significant improvement in quality of life or survival over single agent therapy (Crosby et al., 2000).

Temazolamide, an imidazotetrazinone-derivative analogue of DTIC, is a relatively new alkylating drug with some activity in patients with metastatic melanoma, yielding a 21% overall RR (Richtig et al., 2004). Whereas DTIC requires liver passage for activation, temozolomide undergoes spontaneous hydrolysis for activation and its bioavailability by oral administration is nearly 100%. Because temazolamide crosses the blood-brain barrier, it has been used in patients with brain metastasis with variable

success (Sun and Schuchter, 2001; Richtig et al., 2004). Temozolomide has been found to be used in 60% of stage III and stage IV malignant melanoma cases (Richtig et al., 2004). The side effects of temozolomide are numerous, including leucopenia, thrombocytopenia, anemia, nausea, vomiting, arthralgias, myalgias, increased alkaline phosphatase, fever, infection, rash, diarrhea, alopecia, and parasthesia (Richtig et al., 2004). Several studies with temozolomide have shown equal RRs to DTIC (Newland et al., 1992; Bleehen et al., 1995; Middleton et al., 2000; Bafaloukos et al., 2002; Richtig et al., 2004). Results from the longest study to date (Richtig et al., 2004) found that the most common reasons for discontinuation of therapy in stage IV, with mean duration of treatment 124 days, are disease progression and death, followed by noneligible patients and patients with serious adverse events. The 47 patients evaluated in this study with stage IV malignant melanoma exhibited a mean survival of 14.5 months.

Initial encouraging results using the Dartmouth regimen (cisplatin, dacarbazine, carmustine, and tamoxifen) were associated with overall response rates of up to 50% to 55% in some single-institution studies (Sun et al., 2001) but phase II data have yielded overall median response rates of only 10-20%. Unfortunately, recent phase III trials failed to demonstrate a significant benefit in survival from use of the Dartmouth regimen (Cuevas and Whitman, 2002; Li and McClay, 2002).

Platinum compounds, Cisplatin and Carboplatin have been found useful for palliative effect yet toxicities continue as a barrier to clinical success and methods to ameliorate those toxicities are under active investigation (Richtig et al., 2004). More is known about Cisplatin than Carboplatin. There are similarities as well as differences in efficacy and toxicity, the differences in toxicity being substantial. Cisplatin is much

more emetogenic and somewhat more neurotoxic. Both agents tend to cause preferentially more thrombocytopenia in their respective myelosupression profiles, but cisplatin shows this effect much less than carboplatin. Cisplatin induces apoptosis in a wide range of cell types, and the apoptotic effect may be modulated by cytokines. That effect may vary depending on the cell type and the specific cytokine.

The interplay of combination chemotherapy and the treatment of malignancy is a complex issue. The heterogeneity of malignant melanoma tumor greatly complicates treatment with one agent or a combination of agents for an individual patient. Establishing uniformity and standard therapy for advanced melanoma thus remains very challenging given the variation and heterogeneity of tumor tissue. Beyond these chemotherapeutics, attempts to stimulate the immune defenses of the human have been the aim of treatment in advanced diseased. The next section addresses immunotherapy and combination biochemotherapy agents for treatment in malignant melanoma.

Interferon-alpha

Interferon-alpha (INF-α) was the first human protein effective for cancer treatment (Eton et. al, 199). As the first economically important clinical product for cancer from recombinant DNA technology, interferons have been prototypes for the clinical development of other immunomodulatory and growth-regulatory cytokines. INF-α is approved for the treatment of multiple cancers (Prell et al, 2005). Interferon-α2b was approved by the U.S. Food and Drug Administration (FDA) as adjuvant therapy for patients with stage IIB (≥4 mm primary) or stage III (regional lymph node involvement N1 or N2) disease (Kirkwood et al., 1996). Interferon is usually given to patients for one year with stage IIB or stage III melanoma. Interferon carries a potential side effect

profile which includes a spectrum of flu-like illness, myelosuppression, neurotoxicity (e.g. somnolence, confusion, behavioral changes, and/or seizures), renal insufficiency, and/or cardiac and liver toxicity.

The effect of IFN-α as a single agent or in combination has been widely explored in clinical trials (Ascierto et al., 2005). According to Ascierto and colleagues in their review of international studies for adjuvant therapy of malignant melanoma (2005), critical reading of the major international randomized trials showed that response to IFN-α in terms of improvement of overall survival may not be strictly correlated with the used dosage and that duration of therapy may impact disease-free survival but not overall survival. The author's feel, "the patients' heterogeneity could be an explanation for the discordant data of the international literature."

The majority of these studies started in the 1980's or early 1990's, when accurate staging procedure was not yet available. The adequate surgical treatment, according to the authors, should be considered as an independent variable in the analysis of malignant melanoma adjuvant protocols. Putting together data from all the different studies, INF therapy seems to protect malignant melanoma patients from recurrences during the entire treatment period and prolonged INF therapy seems to improve disease-free survival. The lone positive result regarding overall survival as discovered by Ascierto and colleagues was demonstrated for high-dose INF in a single study (presenting a relatively short follow-up median) and not confirmed in subsequent study from the same authors.

Fluck et al. (2005) conducted a retrospective analysis of 150 consecutive high-risk melanoma patients treated with high-dose INFα2b at a single institution and found similar relapse-free and overall survival data, as previously published from Eastern

Cooperative Oncology Group (ECOG) and Inergroup trials. Their data suggested a transient dose-dependency of the treatment effect and these authors support further prospective trials comparing different dose-distribution patterns in high-dose INF.

INFs pleiotropic properties include inhibition of proliferation and angiogenesis and induction of apoptosis. In some cases, it is also combined with investigational vaccine therapies. Type I INFs also exert immunomodulary effects, which make it an appropriate candidate to combine with cancer vaccines. Fleischman and Wu (2005) examined INF-α to create cancer vaccine cells from a protocol based upon the long-term in vitro treatment of cancer cells with INF-α. This protocol has been used to develop cancer vaccines in mice against B 16 melanoma, RM-1 prostate cancer, and P388 lymphocytic leukemia. The use of the in vitro treatment of cancer cells with IFN-α to create cancer vaccine cells is promising for the treatment of malignant melanoma.

Prell and colleagues (2005) also examined cancer vaccines in mince against B 16 melanoma and found that 50% of mice rejected established B16 tumors following treatment with the combination of a granulocyte macrophage-stimulating factor-secreting tumor cell vaccine (B16.GM) and sub clinical doses of recombinant murine IFN-α delivered at the vaccine site. Similarly, 80% of mice treated with the combination rejected established B16 tumors when recombinant murine IFN-α was given at the challenge site, suggesting that in the latter case its antiproliferative, proapoptotic, and antiangiogenic properties may be involved in controlling tumor growth. In contrast, fewer than 10% of mice rejected the tumors when either one was used in monotherapy. Furthermore, according to the investigators, a 30-fold increase in the frequency of melanoma-associated antigen (Trp-2 and gp 100) specific T cells was observed in mice

treated with the combination when compared with unvaccinated controls. The data show that INF-α combined with a granulocyte macrophage colony-stimulating factor-secreting tumor cell vaccine significantly enhances vaccine potency and may represent a potential new approach for tumor immunotherapy.

In 2004, John Kirkwood MD, director of the Melanoma Program at the University of Pittsburg Cancer Institute and others launched a phase III international trial in patients with stage IIA melanoma with one month adjuvant IFN treatment to compare those who are followed conservatively without chemotherapeutics following surgery in an attempt to reduce recurrence. This trial is still underway (Lange et al, 2004). More randomized trials are needed to determine the effective use of INF beyond the approval given for adjuvant therapy in patients with thick melanomas and in patients with positive lymph nodes.

The use of high-dose IFN remains controversial. There is no indication that extended-duration low-dose INF is significantly better than observation alone in the initial treatment of completely resected high-risk malignant melanoma (Hancock et al., 2004). There is no randomized clinical study showing a survival benefit when IFN is added to any single agent including IL-2 (Lange et al., 2004).

Considering the treatment cost, which is the main goal, disease-free survival, overall survival or quality of life? This is an extremely difficult question. To determine its answer will require further extensive study with special inclusion of the answer given to the patient's viewpoint. Some considerations as to the question must be taken to put order to this field. Considering that low-dose INF is tolerated much better than high dose IFN (about 10% versus more than 70% of cases with grade 3-4 toxicity, respectively), a

prolonged low dose IFN (more than two years) may represent a reasonable opportunity for malignant melanoma patients, also considering its advantageous cost effectiveness (Ascierto et al., 2005). Conversely, considering the improvement of overall survival as the main target of adjuvant therapy, the "wait and watch" attitude remains the only approach to be pursued at present. Ultimately it is a physician's choice.

Interleukin-2

High-dose IL-2 has been extensively studied in metastatic melanoma. Interleukin-2 is a functional cytokine typically reserved for stage IV melanoma. Two large studies have been published using high-dosing schedules (Rosenberg et al., 1998; Lange et al., 2004). Study findings reported overall response rate of 18% and a complete response rate of five percent, with some of the responses being quite durable (Rosenberg et al., 1998). Similar results were reported by Atkins and associates (Atkins et al., 1999; Lange et al., 2004) in 270 patients with metastatic melanoma. Of the 270 patients, six percent achieved complete responses and another 10% obtained partial responses. The striking finding in both studies was that some of the responses were very durable, with responses in some cases being maintained for longer than 120 months.

The substantial toxicity seen with high-dose IL-2 often excludes use in patients with underlying cardiac, renal, or pulmonary disease given increased fluid retention as a major side effect. Administration of IL-2 to these patients can lead to myocardial infarction, renal failure and neurologic changes. The administration of IL-2 requires very frequent and high doses. The severity and nature of IL-2 side effects are related to the dose and schedule used. The patient receiving IL-2 must be hospitalized during administration because of the toxicity profile and its strong tendency to produce

functional alterations in most organ systems. Side effects include transient leukopenia, neutropenia, hypersensitivity reactions, vascular leak syndrome, hyperbilirubinemia, behavioral changes, erythema, and hypothermia. These side effects are associated with severe and life-threatening toxicity. Many low-dose, alternative IL-2 regimens have been evaluated, including low-dose bolus, continuous infusion, and subcutaneous administration. Although these regimens are associated with less toxicity, none has shown a significant objective response rate and none has provided long-term remission.

Combination Immunotherapy

Based on the in vitro synergy between IL-2 and IFN- α in metastatic melanoma, several clinical trials have been conducted combining the two agents (Sparano et al., 1993; et al., 2004). The hope that combined therapy with relatively nontoxic, low-dose, outpatient IL-2 and INF would yield significant clinical activity in metastatic melanoma has not been realized (Richards et al., 1999; Lange et al., 2004).

Biochemotherapeutic Agents

Developed in the 1990s, biochemotherapy combines the administration of multiple chemotherapeutic agents and the administration of biologic response modifiers (INF and IL-2). A recent randomized intergroup trial study led by ECOG evaluated concurrent biochemotherapy in which temozolamide was substituted for DTIC with resulting antitumor activity but low response rates (Atkins et al. 2003). Lack of familiarity of the physicians and nurses in administering the complex biochemotherapy regimen were thought to contribute to the low response rates. Late CNS relapses were found to compromise the benefits of biochemotherapy relative to the high does IFN alone in this study. Atkins et al. (2003) concluded there was no added value attributable to the

ability of temozolamide in reducing the frequency of isolated CNS progress in malignant melanoma. The investigators also concluded that biochemotherapy leads to a very few durable responses and should not be considered a standard therapy. It is suspected that the patients who may benefit from this therapy would need to have an excellent performance status, low-volume disease, or no previous treatment with INF. Finding such patients to enter into these trials is quite a challenge given that many have already been started or tried with INF and underlying diseases states are fairly common among the age-group to which advanced melanoma strikes.

Previous phase II studies using biochemotherapy (combination of platinum-containing chemotherapy with IL-2 and IFNα) have shown response rates of about 50% (Lewis et al., 2005). However, a site of frequent relapse is the central nervous system (CNS). Temozolomide has equivalent activity to dacarbazine, but it has the advantage of CNS penetration. Lewis reported results of a phase II study using a novel biochemotherapy regimen containing temozolamide, cisplatin, decrescendo IL-2, INFα, and GM-CSF in the treatment of stage IV melanoma. Seventy-one patients with histologically confirmed metastatic melanoma were enrolled between June 1998 and October 1999. Prior chemotherapy or IL-2 was not permitted. The median age was 54 (range 22-72). Twenty-one patients (30%) had a history of treated brain metastases. Patients received temozolamide 150mg/orally days 1-5, cisplatin 30 mg/m2 IC days 1-3, INFα 5MU/m2 SQ on day 1-5, and IL-2 was administered in a decrescendo fashion according to the following schedule: day 1: 18MU/m2 continuous IV infusion over 6 hours, day 2: 18 MU/m2 continuous IV infusion over 12 hours; day 3: 9 MU/m2 subcutaneously every 12 hours, day 4: 4.5 MU/m2 SQ. Patients were also given GM-CSF

250 microgram SQ days 6-25. The cycles were repeated every 4 weeks. Partial responses were seen in 10 of the 71 patients (14%) with a median duration response of 9.4 months. There were no complete responses. The median survival for all patients was 8.6 months. The investigators suggest further studies of this novel biochemotherapy regimen are not indicated and suggest other schedules that incorporate temozolomide and/or GM-CSF and further studies to define the optimal method of delivering IL-2 should be pursued.

In conclusion, the agents available for malignant melanoma vary in response rate, survival outcomes, and complexity and cost (Crosby et al., 2000). "For many anticancer agents, the paucity of data prohibits formal dosing recommendations, and most guidelines remain empiric" (Eklund et al., 2005). At present, treatment for advanced melanoma is neither standard nor uniform. There is not a diagnostic test that can provide assurance to the treating physician which accurately predicts drug resistance. Treatment for advanced melanoma at present is derived from an integration of clinical experience, evidentiary medicine and physician intuition.

In the future decision making may be guided more so by evidence and individual in vitro tumor testing. In that regard, the field can move forward. There is currently research being pursued in administration of in vitro-activated and –expanded autologous tumor-reactive T cells, currently one of the few immunotherapies that can induce objective clinical responses in significant numbers of patients with metastatic solid tumors. This work will be highlighted in the last chapter. The following table lists 2003 AJCC recommendations for systemic treatment (table 8). Following table 8 is a discussion and table listing the surgical management of melanoma stage III and IV.

Table 8 Systemic Treatment for Melanoma (Lange et al., 2004)

Adjuvant Therapy
Primary melanoma ≥ or with regional lymph node involvement should be considered for treatment with high-dose IFN after appropriate surgery. High-dose IFN is the only adjuvant treatment shown to improve disease-free survival and possibly overall survival. These patients may be offered enrollment in clinical trials of adjuvant therapies, either in Phase II setting or in a Phase III trial with IFN or observation as a control. Standard therapy for patients with resected melanoma < 4 mm and pathologically negative regional lymph nodes remains observation only. However, patients with ulcerated primary lesions thicker than 2 mm might be considered for high-dose INF or a clinical trial, because their survival rate approaches 50%. Some adjuvant clinical trials enroll patients with melanoma was than as 1.5 mm.
Treatment with Advanced Disease
Because there really is no standard therapy for patients with metastatic melanoma, all patients should be considered for clinical trials. Patient with ECOG performance status of 0 and normal heart, lung, and kidney function should be offered high-dose IL-2, preferably in high-dose Il-2 based clinical trials, as this therapy offers a five percent chance of achieving durable remission. Patients who do not meet the criteria for high-dose IL-2 therapy can be considered for clinical trials of less toxic new treatments. Patients without assess to a trial can be offered either DTIC, with or without tamoxifen, or temozolomide, with or without thalidomide. Combination chemotherapy, such as Dartmouth regimen or CVD, offers a slightly better chance of achieving durable remission than combination chemotherapy; however, patients must understand that this approach is unproven, and the chance of achieving durable remission with any of these treatment options is small.

MANAGEMENT OF LOCOREGIONAL RECURRENCE

Local Recurrence of Malignant Melanoma

Local recurrence rates after appropriate wide local excisions are low. With long term follow up of intermediate thickness lesions in the Intergroup Melanoma Trial, 2.1% to 2.6% of patients had a local recurrence (Balch et al., 2000; Lange et al., 2004). Patients with fairy high risk for local recurrence are those with thick primary lesions (≥4 mm) and those with histologic ulceration, desmoplastic features, or a high mitotic rate.

Local recurrences can be a sign that patients have or soon will have systemic disease. When local recurrence is detected, the minimal systemic work-up includes chest radiograph and should include serum LDH. If abnormalities are found CT scans, PET scans are indicated (Lange et al., 2004).

In transit metastases appear as identifiable tumor nodules in the subcutaneous or cutaneous tissues between a primary site and it's nearest draining basin. Approximately 2% to 4% of patients eventually have in-transit disease after excision is performed for localized primary melanoma (Essner et al., 1999; Lange et al., 2004). As with local recurrence, in-transit disease can be a harbinger of impending systemic disease; therefore, patients with in-transit disease should undergo a staging evaluation. If limited in-transit lesions are present and they are amendable to excision, wide local excision with negative margins is the treatment of choice (Lange et al., 2004).

The current AJCC surgical guidelines for melanoma recommend a two-stage excisional biopsy of the suspected lesion with a narrow margin of normal skin (Sober et al., 2001; Roberts, 2002; McKenna et al., 2004). This allows for confirmation of the diagnosis and permits the second stage of wider local excision to take in account Breslow thickness when planning surgical margins. Current evidence suggests that a 1-cm clinical margin around the lesion is adequate for melanomas with a Breslow thickness of <1 mm and a 2-3 cm margin for tumors 1-4 mm in depth (Veronesi, et al., 1988; Veronesi et al., 1991; Balch et al., 1993; Cohn-Cedermark et al., 2000; McKenna et al., 2004). General consideration is no additional advantage of margins for melanomas of > 4 mm thickness. Justifications for wide local excision include: removal of lymphatic tissue surrounding the lesion which may have become directly permeated by melanoma cells (Handley 1907;

McKenna et al., 2004); removal of adjacent epidermal melanocytes which may have undergone a field change effect and therefore have the potential to become malignancy at a later date (Cochran, 1971; Wong, 1970; McKenna et al., 2004); and the removal of micrometastases before they can grow into clinically detectable metastatic deposits (Kelly et al., 1984; Eedy, 2003; McKenna et al., 2004).

Patients that have a recurrence in a previously undissected node basin should have a complete node dissection plus staging workup as above. Adjuvant radiation therapy should be considered. The indications for isolated limb perfusion are limited. Limb perfusion with hyperthermia and melphalan has never been shown to be associated with improved survival, but has a role in securing local and regional control for patients with unresectable local recurrence or a large volume of in-transit disease. This procedure is performed routinely by the surgeon in the Yale Cancer Center Melanoma Unit with success of local and regional control in over 60% of surgical cases.

The recent revision to AJCC staging system for melanoma proposes parameters for survival prediction. Members of the Committee feel that "patients can be identified who have relapse rates of 50% or more" (Kirkwood et al., 1996; Lange et al., 2004). All stage III and IV patients should be strongly considered for lymphadenectomy in patients with ≥ four positive nodes of extra-nodal extension.

Table 9 AJCC Guidelines for Surgical Management of Stage III and IV Melanoma (Lange et al., 2004)

Pathologically positive regional nodes →	**Complete lymphadenectomy**
Local recurrence or in-transit metastases, limited →	**Complete resection, 1 cm margin where possible**
Local recurrence, or in-transit metastasis in an extremity, extensive or symptomatic →	**Consider limb perfusion for regional control**
Distant metastases: solitary or symptomatic →	**Complete surgical resection if not unduly morbid**

Although melanoma is a relatively radiation-resistant tumor, palliative radiation therapy may alleviate symptoms. Retrospective studies have shown that patients with multiple brain metastases, bone metastases, and spinal chord compression may achieve symptom relief and some shrinkage of the tumor with radiation therapy (Rate, Solin and Turrisi, 1988; Herbert et al., 1991; National Cancer Institute, 2002). The most effective dose-refraction schedule for high-dose-per-fraction schedules are sometimes used to overcome tumor resistance. An on-going phase I/II clinical trial is evaluating adjuvant radiotherapy plus INF in patients with recurrent melanoma (Deconti and H. Lee Moffitt Cancer Center Research Institute, 2001; National Cancer Institute, 2002).

To summarize, the overall current standard treatment options for malignant melanoma are: 1) Resection of isolate single or localized metastases from skin, visceral, or brain sites in selected patients, which is sometimes associated with prolonged survival, and 2) Palliative radiation therapy for bone, spinal chord, or brain metastases, and 3) Palliative biologic therapy and/or chemotherapy in phase I/II clinical trials, and 4) Palliative treatment with IL-2 or INF, which can occasionally result in prolonged survival, and 5) Isolated hyperthermic limb perfusion for extremity (Lange et al., 2004).

Conclusion to Chapter 1

It is clear that melanoma shows low response rates to immunotherapy and chemotherapy. Both forms of therapy appear to kill melanoma by induction of apoptosis, so it is possible that resistance of apoptosis may underlie the low responses to these forms of therapy. Much is already known about the agents that may sensitize melanoma to apoptosis and combining these with chemotherapy and/or immunotherapy provides new accepted but still experimental treatment for patients who have been rendered clinically

free of disease by surgical resection but are at high risk for recurrence and in selected patients with advanced but still limited disease. In general there seems to be a correlation between the ability of melanoma vaccines to stimulate anti-melanoma cellular or antibody immune responses and improved clinical outcome (Bystryn, 2002). The potential promise has been demonstrated by a series of encouraging small trials such as the randomized trial by Bystryn (2002) and others (Hsueh et al., 2002; Bystryn and Reynolds, 2005).

In order that vaccines are effective they must contain antigens that can stimulate tumor-protective immune responses and indeed some of these antigens must be present on the tumor to be treated. Unfortunately for malignant melanoma these antigens are not known. There is a sense of impatience regarding the development of melanoma vaccines. Several strategies are underway to construct polyvalent vaccines that contain a broad array of melanoma-associated antigens. The good news is that clinical trials have shown that vaccines are safe to use and have much less toxicity than current therapy for melanoma. According to Bystryn (2002) there is a growing body of evidence that suggest that vaccines can be clinically effective in melanoma treatment. This evidence is mounting in a body of research already underway that incorporates correlations between vaccine-induced antibody or T-cell responses and improved clinical outcomes, clearance of melanoma markers from the circulation, improved survival (compared to historical controls) and most convincingly, two randomized trials in which the recurrence-free survival of vaccine-treated patients was significantly longer than that of control groups (Bystryn, 2002). The challenge of ideal individual therapeutic management for advanced

melanoma is to construct and effectively employ favorable clinical response and determination as to how best monitor that approach effectively.

Immunohistochemistry serum identification has been shown to distinguish poorly differentiated amelanotic malignant melanoma from tumors of obscure origin. Current research focuses on the prognostic features of biomarkers for solid tumors, including melanoma (Rimm et al., 2001; Berger et al., 2003; Parker et al., 2003; Berger et al., 2004; Cloven et al., 2004; DeVita et al., 2004; Freuhauf, Kyshtoobayeva, and Yu, 2004; Kluger et al., 2004). The evaluation of CKit expression of solid tumors may have predictive utility if these results can be independently confirmed. (Freuhauf et al., 2004). The K1-67 proliferation rates and p53 expression in minimal deviation melanomas may represent a distinct entity and may demonstrate prognostic power in malignant melanoma (Chorny, 2003). The potential value of biomarkers in identifying effective therapy has been demonstrated by Freuhauf et al., in their work with BCNU, cyclophosphamide, ifossfamide, temozolomide and dacarbazine and tumors that expressed Kit/CD117. These studies provide optimism for further research in malignant melanoma therapeutics.

Tissue micro-array construction can provide a highly efficient, high-throughput mechanism for evaluation of protein expression in large cohorts and has the potential for allowing quantitative assessment useful in melanoma. Its validation potential was shown recently using the AQUA to validate potential tissue biomarkers as a valuable prognostic tool for management of malignant melanoma (Berger et al., 2004).

Given poor therapeutics for metastatic melanoma, innovative diagnostic identification testing may yield benefit in guiding therapeutic choice for the treating physicians. The exposition of malignant melanoma is far from well-defined. Tumor

heterogeneity plays havoc with histopathology and cellular behavior. The heterogeneity vastly interferes with effective drug response predictability. The identification of in vitro methods found to isolate parameters of heterogeneity may elucidate drug resistance and contribute to therapeutic drug determination.

In vitro testing methods are an avenue to learning more about drug resistance and may in fact yield benefit in prognostics and therapeutics and thus become useful to the clinicians who treat malignant melanoma. The in vitro drug-response assays will be reviewed and the relevance of their use in Chapter 2. The focus of the chapter is given to the extreme drug resistance in vitro assay conducted at Oncotech Inc. Laboratory.

Consideration is given to these testing methods if they are likely to fundamentally affect clinical choice if their utilization is found to outweigh therapeutic decision without their use. The clinician who devises treatment must be appraised of results from clinical trials, the physiology particular to the melanoma cell cycle in relation to extreme drug resistance and remain proficient in the efficacy of current and available testing methods that may enhance decision making and determine the therapeutic regiment for each individual patient. This determination must be balanced with the needs of the individual patient, their present disease state and their specific tumor characteristics

CHAPTER 2: IN VITRO DRUG RESPONSE ASSAYS

The original research contained in this document, which will be presented in Chapter 3, considers the use of drug resistance testing (extreme drug resistance) in the treatment of malignant melanoma. The research examines whether the use of this type of test to guide the choice of therapeutic drugs would be likely to yield fundamentally different clinical choices than are currently made without their use. The in vitro testing is commercially available for physicians who treat malignant melanoma.

This chapter will review potential advantages of extreme drug resistance (EDR) testing, its limitations, and cost-effectiveness. As the research undertaken here focuses on the use of EDR tests, it is relevant to discuss the history of in vitro testing, describe its variants, and discuss the available methods within its class. A discussion of these issues may be is relevant to any potential study involving EDR in vitro testing and is presented in this chapter.

History of In Vitro Testing

In vitro tissue micro array testing is a diagnostic technique that can help identify the drugs to which a given patient's tumor may yield resistance. In vitro cell culture drug resistance testing (CCDRT) is purported to correlate with response to chemotherapy and/or with patient survival (Weisenthal and Nygren, 2001). Data from several studies have indicated that chemoresistance assays may be used to select patients who might benefit from an individually adapted cytostatic therapy (Ugurel et al., 2003).

There are two basic types of in vitro assays, those which examine chemosensitivity and others which consider chemoresistance. Chemosensitivity refers to the ability of an agent to kill target cells. Chemoresistance refers to the ability of target

cells to withstand exposure to an agent. Each type of test will be discussed to distinguish among them in consideration of the history of this area of research. An emphasis will be placed upon in vitro extreme drug resistance testing given the focus of this study.

The origin of in vitro drug response testing stems from the work of Erlich and Pasteur who evaluated agents of microbial and synthetic origin on the growth of cultured microbes in the 1870's (Albert, 1965). Erlich, who coined the term "chemotherapy", emphasized the need for agents that were selectively toxic. Following this seminal work, Fleming's discovery of penicillin in 1929 introduced the modern era of culture and sensitivity testing. Subsequent discoveries of bacterial antibiotic resistance mechanisms and improvements in tissue culture technology paved the way for translating this approach to oncology. The first attempts to evaluate surgically resected tumors for drug response did not occur until the early 1950's.

In the mid-1950's Black and Spear (1953; 1954) were the first to report the use of an in vitro assay to predict patient response. Their studies compared the in vitro activity of aminopterin with its clinical response. Their assay technology was based on the colorimetric detection of viable cells using a substrate for mitochondrial succinate dehydrogenase. Although the predictive accuracy of their results was not particularly strong, they introduced the development of the clonogenic stem cell assay (Black and Spear, 1953; 1954; Freuhauf, 2002).

The 1970s brought in vitro testing of solid tumors into the mainstream (Salmon et al., 1978). Although several methods had been developed since the 1950's to determine in vitro drug sensitivities of human tumor cells to various anticancer agents (Bird et al.,

1987 and Selby et al., 1983), the results of the studies conducted in the 1970's indicated that there were still serious technical issues to overcome (Clark and Von Hoff, 1984).

One such issue was the protracted turn-around-time which exceeded a time frame necessary for its inclusion in the treatment determination by the physician. This rendered the in vitro methodology less effective for the treating physician as a diagnostic tool, particularly for malignancies where life span was short.

The cost of testing was also a detriment to utilization. Medical insurance companies refused to consider reimbursement for patients since in vitro testing was deemed experimental, having not yet been shown to correlate with patient outcomes. Moreover, there were few patients willing to pay themselves for a test that was not shown to be effective, accurate, or timely. It seemed at the time that the obstacles inherent to in vitro drug testing might be too formidable to overcome.

Despite this somewhat discouraging start, in the 1980s several laboratories continued to pursue this method of tumor tissue testing in the laboratory. Ultimately, advances in the testing technologies overcame many of the technical problems of the earlier systems. Assays were developed that yielded results for most cases in less than a week. This was achieved, in part, by identifying factors that prevented tumor growth in vitro and adjusting the assay methodology in an appropriate direction. Other adjustments in the assay procedure that shortened delivery time were developed by comparing information across clinical trials to assess the relative advantages and disadvantages of different techniques (Weisenthal et al., 1985; 1991). The shorter turn around time that resulted for delivery of assay results made the use of the tests in clinical applications viable.

Divergent assay endpoints were generally found to provide comparable predictive accuracy or value, which for this discussion are interchangeable. One particularly notable finding that emerged from these comparisons was that regardless of the endpoint employed, in vitro drug response assays were most reliable for accurately identifying drugs that were unlikely to be effective rather than for choosing drugs that would cause tumors to shrink. The negative predictive accuracy (NPV) was generally 90% to 99% while the positive predictive accuracy (PPV) was only 50% to 70% (Weisenthal et al., 1985; 1991).

Test Accuracy, Sensitivity and Specificity

There are several concepts which are related to each other; sensitivity, specificity, positive predictive value, negative predictive value, and Bayes' Theorem. For the relationship between all of these statistical concepts with the exception of Bayes' Theorem, Chu (1999) provides an excellent overview. Ashby and Smith (2000) provide a discussion of Bayes' Theorem in a medical decision making context. Stanford (2005) provides a number of alternative formulations of Bayes' Theorem and explains the interrelationship between these measures. The results of a typical test can be stratified using a four way table as presented here. Those who are characterized by the presence of the disease may either test as a true positive or false negative. Similarly, those with the disease absent may test as a true negative or a false positive. Where the test results lie for a particular sample relative to the truth determines the values of the probability measures discussed here.

	Disease present	Disease absent
Test Positive	True positive	False Positive
Test Negative	False Negative	True Negative

Test results are ideally related to the truth. End users of tests are interested in knowing at an intuitive level how accurate results are, but the question is accurate relative to what? In some cases, there are procedures available that can determine the true prevalence of disease for purposes of comparing test results. In other cases, this is not possible but there is a test or method of determining presence of disease that has been established as the most reliable method. In the testing literature, the gold standard refers to a procedure that determines a true diagnosis but the term is also used in this literature to refer to the most accurate test or procedure available (Chu, 1999; Crosby et al., 2000).

Sensitivity is defined as the proportion of patients with the disease who have a positive test, that is, the probability that a person with a disease will test positive (Alberg et al., 2004). Specificity is defined as the proportion of patients without the disease who have a negative test or true negatives or the probability that a disease-free individual will test negative (Chu, 1999). For calculating these measures, one is summing observations that fall into the two columns of the table to enter into the relevant denominator of the ratio. The concept of the gold standard is relevant in these calculations in the external determination of who has the disease and who does not. The results of the test under consideration are compared to the gold standard to determine the accuracy of test results.

Overall accuracy is the probability that an individual will be correctly classified by a test. This is calculated as the sum of the true positives plus true negatives divided by the total number of individuals tested. Thus, for a test to be accurate, it must be both highly sensitive and highly specific. Decreasing false negatives increases sensitivity. Reducing false positives, increases specificity. While a highly sensitive and highly specific test is desirable, there may be a trade-off between sensitivity and specificity. As will be discussed shortly, there is a large trade off in tests for drug sensitivity versus specificity (resistance) in the case of malignant melanoma. Chu (1999) provides mathematical examples of cases where such tradeoffs exist.

As with any other statistical concept, sensitivity and specificity are estimated from samples of patients and thus, the measures are subject to sampling variation. It is common that ranges of 95 percent confidence intervals are reported to reflect the variance inherent in the measures (Chu, 1999). Since variance as a statistical concept decreases with sample size, confidence intervals become more tightly banded as sample sizes increase.

Positive and Negative Predictive Values

Sensitivity and specificity convey information regarding how close to the reference standard for a disease a test will come. However, once a test has been performed, sensitivity and specificity do not reveal whether a positive (negative) result truly indicates the presence (absence) of the disease. That information is given by the predictive values.

The positive predictive value, PPV, is defined as the proportion of patients with a positive test that has the disease or true positive. Negative predictive value, NPV, is

defined as the proportion of patients with a negative test that does not have the disease or true negative. These measures answer the question of how accurate the test is conditional upon its result. The denominator for calculating these two probabilities are formed by summing across the rows of the two-way table presented in the section on sensitivity and specificity.

For constant rates of sensitivity and specificity, it can be shown that predictive values vary with disease prevalence. Chu (1999) contains hypothetical examples where this point is demonstrated. The prevalence of a disease is defined as the proportion of the population that has the disease at a given time. In holding sensitivity and specificity constant, one finds that as prevalence falls, the PPV decreases and NPV increases. As prevalence rises, PPV increases and NPV decreases.

Bayes' Theorem

Bayes' Theorem is a mathematical formula that relates conditional and unconditional probabilities to each other (Ashby and Smith, 2000). The theorem has usefulness in the medical field because it can be shown to relate measures of sensitivity and specificity in combination with prevalence rates to predictive values. Where local rates of disease prevalence differ from those in the general population, it can be used to calculate correct (local) rates of positive or negative predictive values.

To demonstrate this point, a formula from the Stanford Encyclopedia of Philosophy (2005 eqn. 1.3) is reproduced here.

$$\mathbf{P}_E(H) = \mathbf{P}(H)\mathbf{P}_H(E) \,/\, [\mathbf{P}(H)\mathbf{P}_H(E) + \mathbf{P}(\sim H)\mathbf{P}_{\sim H}(E)]$$

Summarizing their explanation, in this formulation, H is the event of having a disease and E is the event of testing positive for it. $\mathbf{P}_E(H)$ is the probability of a disease given a

positive test result (positive predictive value). P(H) is disease prevalence. P(~H) = 1 - P(H) or the proportion of the population not having the disease. $P_H(E)$ is the test sensitivity. $\mathbf{P}_{\sim H}(\sim E)$ is the test specificity. If P(H), $P_H(E)$, and $\mathbf{P}_{\sim H}(\sim E)$ are known, then $\mathbf{P}_E(H)$ can be calculated. This says that if prevalence, sensitivity, and specificity are known, then the probability of a disease (positive predictive value) given a positive test can be calculated.

In cases where a clinician encounters a population of patients with a prevalence that differs from the population of a study, as long as the specificity and sensitivity of the test have been reported, a positive predictive probability (PPV) can be calculated using the above formula. Similar formulas can be used to calculate the negative predictive probability (NPV).

There are many variants of Bayes' theorem. For example, the equation presented above could be mathematically manipulated to allow the calculation of specificity if sensitivity, prevalence, and positive predictive value were known quantities. Other forms of Bayes' Theorem are written in a form that relates to the issue of how prior odds of clinicians in making diagnoses and the final likelihood of making a correct diagnosis can be impacted by the use of tests with known stochastic properties. For the purpose of this chapter on testing, a further discussion of those additional considerations is unwarranted.

Accuracy of In Vitro Assays

The application of these statistical concepts to drug-response tests yields results that are understandable from an intuitive perspective. The systematic variation of in vitro drug-response tests in terms of their sensitivity and specificity is not very surprising when the complexity of drug delivery to the patient's tumor in vivo is considered. In

vitro assay systems can dependably deliver the drug being studied to the tumor cells in culture; however, numerous problems arise with drug delivery in vivo. This can be found by consideration of some of the complications of in vivo cancer treatment. After intravenous administration, chemotherapy agents are subjected to significant individual differences in biotransformation and biodistribution. Biotransformation differs among patients in part as a function of their enzymatic haplotype. Analysis of the impact of single nucleotide polymorphisms on drug activation is an emerging area of pharmacogenetics that may lead to patient specific drug dosing (Kim et al., 2001). Recent data on single nucleotide polymorphism has also provided new insight into the genetic basis for why some patients rapidly inactivate drugs, while others suffer greater toxicity by virtue of their slower drug metabolism (Roses, 2001). Individual differences in drug metabolism that might prevent an active form of the drug from reaching the tumor in vivo cannot be modeled using the current in vitro assay systems.

Pharmacodynamic activity ultimately depends on biodistribution of active drug species to the tumor bed through the tumor's blood supply. A great deal of evidence has emerged supporting the notion that tumors of a given type and grade may possess a wide range of microvessel densities. Angiogenesis has emerged as an important and new prognostic factor, as well as a new target for cancer treatment (Chen et al., 2001). Multiple effects usually occur in the cell cycle, during and after the exposure to a drug, while treated cells flowing through the cycle encounter G(1), S and G(2)M checkpoints (Lupi et al, 2005). Unfortunately, individual differences in tumor vascularity are not accounted for by current in vitro drug-response assays, adversely impacting on their positive predictive capability. Current in vitro assays lack the capability to account for

these critical pharmacodynamic aspects of drug delivery, making it difficult for them to accurately predict in vivo 'chemosensitivity.' On the other hand, while these pharmacodynamic factors do not accurately predict that a drug will work, they correlate with drug resistance. If the tumor sample is completely resistant after supraoptimal drug exposures in vitro, then suboptimal in vivo delivery resulting from poor tumor vascular supply and/or rapid drug inactivation will most likely result in treatment failure.

Some examples of poor positive predictive probabilities for in vitro assays can be found in the literature. In one trial conducted by the Southwest Oncology Group, only 23 of 168 patients (14%) received therapy selected by an in vitro assay. The remaining patients in the trial had tumors that either did not form colonies in vitro or did not show sensitivity to any agent. The overall negative predictive value for over 2500 correlations was 91% (McGuire et al., 1995).

In vitro tests for drug response provide a bridge between the current empirical approach to chemotherapy and the future era that will focus on treatment tailored by biochemical fingerprinting. While cancer cell biology and critical signaling pathways are at present being researched and hold great promise, the field is still fairly nascent. Even when specific pathways are identified and appropriate drugs are administered, resistance often emerges.

In this context, the main advantage of in vitro drug-response assays is their determination of the effect of drug action on cancerous cells. Drugs either induce apoptosis or they do not. The measurement of a specific drug target may not account for all the steps required for drug efficacy. For example, tamoxifen is expected to be effective as an initial treatment in patients with steroid receptor-positive breast

carcinoma. However, co-expression of HER2 can diminish breast cancer sensitivity to tamoxifen (Houston et al., 1999). Thus, a myriad of pathways may be interacting simultaneously to impact on drug entry into a cell, drug movement to its site of action, and the effect of the drug to induce cell death.

In vitro tests make it plausible to determine if the process of drug action and cellular response is intact or not. The oncologist may take advantage of these findings in two ways. First, because these assays offer identification of ineffective drugs, such agents can be avoided. Second, while the accuracy of predicting that a drug will work is limited, agents to which the tumor demonstrates low resistance have been found to be more effective than those found to be extremely resistant in vitro. Integration of pertinent clinical factors together with in vitro data on tumor response to agents defined to be ineffective for the patient's tumor type may offer the best outcomes for patients with breast, ovarian, or melanoma cancer at this time.

In the 1990's to present, the technology of in vitro testing has improved and utilization has increased. It is a method now in use by a number of oncologists and institutions as its pronounced ability to identify drug resistance accurately has gained attention and respect in the field of oncology. Additional assay methods are under use and study as proponents of the technique attempt to move it forward towards routine utilization among treating oncologists. There are a variety of methods that have come and gone which have paved the way for those most commonly used today.

ASSAY METHODS FOR DETERMINING EDR

Methodologies for determining extreme drug resistance

The advent of reliable in vitro drug-response assays has raised the possibility of selecting effective anticancer agents to be used either alone or in combination to treat a patient's individual tumor. In this setting, identification of agents with an extremely low probability of response makes it possible to eliminate the use of those agents and thus their potential for adverse events. A number of methods have been used to investigate the sensitivity of tumors and tumor cell lines, including clonogenic, differential staining cytotoxicity assay; colorimetric, rapid H-thymididine incorporation assay; and chemotherapeutic treatment of athymic nude mice with human tumor xenografts. The following are alternative methods and their commonalities across the techniques and their different endpoints.

The study by Black and Spear in 1954 was a relatively small study however it made a major contribution towards the development of in vitro assay systems. Black and Spear found that predicted accuracy of tumor response in the laboratory setting was favorable regarding resistance but weak regarding sensitivity. Their work "foreshadowed some of the technical hurdles that would confront subsequent investigators" (Freuhauf, 2002). First, their assay endpoint measured the metabolic activity of both cancer and normal cells, making it difficult to distinguish between drug effects on the normal versus cancer components. In addition, because they employed tumor segments, the contribution of the malignant component to the endpoint signal varied from patient to patient, making it difficult to standardize the system and compare results between patients. Finally, their findings suggested that the accuracy of their system to predict the treatment response (the positive predictive value), was not as great as the negative predictive value to predict treatment failure (Freuhauf, 2002).

This system eventually evolved into the MTT assay system utilizing tetrazolium dye and was incorporated into the National Cancer Institute cancer drug discovery and developmental program. Although the National Cancer Institute program now uses the sulforhodamine B assay in its drug screening program with cell lines, the MTT assay continues to be evaluated as a predictive assay in the clinical setting.

The clonogenic assay was the second significant attempt to develop a reliable in vitro drug-response method. Originally developed by Puck and Marcus (1955) to assess the impact of radiation on tumor cell growth, it was applied in the context of drug impacts on human tumors in the mid-1970s. Early clinical studies led to a great deal of enthusiasm and high expectations for the clonogenic assay. Commercial laboratories sprang up to offer this testing but unfortunately this was premature.

When problems with low rates of assessability emerged, the very concept of using in vitro methods to predict the drug response of patients was questioned. Despite this setback, alternative approaches taken during the ensuing years improved assessability and overcame important quality control problems.

The next substantial progress in resolving these technical difficulties with testing assays for human tumor cells was due to Anne Hamburger, Sydney Salmon, and their colleagues, who developed a technique to selectively grow human tumor cells in vitro (1977). By 1983, an editorial (Von Hoff, 1983) was published in the *New England Journal of Medicine* entitled "Send the Tumor for Culture and Sensitivity." The article proposed the concept of routine testing of tumors for in vitro drug sensitivity in much the same way that bacterial isolates for antibiotic susceptibilities are tested (Von Hoff, 1983).

Common Basic Steps of In Vitro Assays

Although the techniques for testing drug sensitivities of tumor cells differ, each employ four common basic steps: (1) isolation of cells, (2) incubation of cells with drugs, (3) assessment of cell survival, and (4) interpretation of the result (Brown and Markman, 1996). The quality of assays is based on the assessability rate (i.e. the percentage of specimens submitted that can be successfully assayed), the number of tumor types that can be assessed, turnaround time, and clinical relevance of the results.

The first step, isolation of cells, is achieved in solid tumors by excision and disaggregation (mechanical or enzymatic) to liberate the tumor cells. In the second step, an incubation period is required to allow the drugs to act on the tumor cells. This can last from hours to weeks. Most assays take four to five days. In the third step, an end point for the experiment is chosen and used to assess cell survival. The choice of an assay end point to evaluate drug effects distinguishes the various methods. The final step is used to tabulate, report, and interpret the test results.

The in vitro drug response testing method, like prognostic and predictive tumor marker testing methods, must be validated clinically by correlations with patient outcomes. The method should be robust enough to provide information for a variety of chemoresponsive tumor types. The turnaround time for the procedure should make results available in a clinically relevant time frame. Test results should be easily understood and applied. The benefits of using the test information should be clinically relevant and cost effective. These issues should be addressed when evaluating the clinical utility of specific assay techniques. Many of the results are reported back to the ordering surgeon and oncologist as drug sensitive, resistant, and intermediate. Others have developed this further by calculating a drug sensitivity index to put the result into

the context of other similar assay results or by calculating a probability of response, taking into account expected response rates where these are known (Kern and Weisenthal, 1990; Parker et al., 1992).

Classification of Array Testing

The major distinction among the differing assay methods is the end point used to measure cell viability. Assay end points include colony growth from a single stem cell, incorporation of tritiated thymidine and microscopic examination of cells with vital dyes, mitochondrial enzyme activity, cytosolic esterase activity, and adenosine triphospate content. Given the variety of assay types, it is remarkable that the predictive accuracy identification of chemosensitivity for most of these approaches appears to be at least 90% (Kern and Weisenthal, 1990; Parker et al., 1992).

Chemosensitivity Assay Testing Methods

The majority of chemosensitivity assays currently in use provide a measure of cellular proliferation; however, some have been developed to assess metabolic activity, dead cell percentage, or apoptosis. The utility of the assays, and the determination of which assay to use, is truly dependent upon the cytotoxic drugs being tested. This overview will reveal that chemosensitivity assays yield substandard results compared to chemoresistant assays.

The MTT assay provides a simple, rapid, semi-automated technology that is readily reproducible between laboratories. Use of MTT assays to predict responses for hematologic malignant conditions appears promising when cancer cells constitute more than 80 to 90 % of the population of cells (Weisenthal and Nalick, 2004). However much of the work is based on the determination of tumor cell percentages at the beginning of

the incubation period, a value that could change significantly after four days in culture. For solid tumors, the situation is more of a problem because of the predominance of stromal cells in the tumors. Another drawback is the finding that some cells that have been damaged lethally and have lost their dye exclusion capability still can metabolize MTT (Weisenthal and Nalick, 2004). This finding suggests that the MTT assay is best applied as a proliferation inhibition assay.

The clonogenic assay or human tumor clonogenic assay (HTCA) and its derivatives evaluate the ability of chemotherapeutic agents to inhibit tumor stem cell proliferation in agar. The cancer cells proliferate readily in agar, an anchorage-independent environment. In clonogenic assays, solid tumors are disaggregated into single cell suspensions with scissors and by passing the fragments through steel mesh and high-gauge needles. The cells are washed, treated with the drug(s) being tested for one hour, and plated on agar with growth media. After two weeks, the number of colonies that have grown from the treated cells is compared with the number from untreated control cells. One significant problem with this method has been the difficulty of completely dissociating tumor specimens into single cells. If cell clumps are plated initially, they can be misread on day 14 as colonies. A number of studies have been done using clonogenic assays, but many technical limitations hinder their routine use in clinical trials: labor intensiveness, high cost, and poor standardization due to low plating efficiencies (approximately 58%), clump artifact, and long assay duration (Kern and Weisenthal, 1990; Berger, 2003). However the use of chromomycin A3 as a control to detect clumping minimizes this problem. This method continues to be evaluated in clinical trials with assessability rates of 75% to 85% (Monk et al., Berger, 2003).

Tritiated thymidine uptake also can be assessed in the agar-based clonogenic assay system. The use of tritiated thymidine as an end point was developed by Kern et al. (1985) to eliminate the problem of discriminating between true colony growths from a single cell versus a clump of cells plated at the outset secondary to poor dissociation (Kern et al., 1985, 1990, Sondak et al., 1984). The protocol for the 3H-Thymidine-incorporation (TIA) assay is quite similar to the HTCA, except for the method used for evaluation between treated and control cells. The difference is that here, the small clumps are preferred to maintain cell-cell interactions. Tumor suspensions are exposed to drug for three hours to five days, depending on the study. Tumor cells are exposed to tritiated thymidine during the final 48 hours of the assay, which is incorporated into the DNA of reproducing tumor cells during the S-phase. The incorporation of labeled thymidine is determined after liquefying the agar-cell suspension by heating the culture plates, harvesting the cells into glass-fiber filters with a micro-harvester, and counting the radioactivity with a liquid scintillation counter. Unlike the clonogenic assay, some authors suggest that small clumps are acceptable in this assay as they maintain the interactions between the cancer cell and the surrounding stroma. However one must realize that this also presents the problem of extraneous "noise" from normal cells capable of incorporating 3H-thymidie during S-phase. In addition to eliminating clump artifact, the TIA renders reliable results in 85% of tumor explants examined, and can be completed within seven days of surgery (Monk et al., 2002; Berger, 2003).

The percent inhibition of cell proliferation is determined by comparing the treatment group's counts per minute with the untreated control groups. The relative response for each patient to a given substance is determined by comparing that agent's

action (percent inhibition) on the tumor with the median percent inhibition and standard deviation from a population of tumors. Thus, each patient's tumor response in the assay is relative to a large group (>800) or tumors. Although this method has a reasonable (72%) overall predictive accuracy (PPV), it excels (92%) at predicting drug resistance ((NPV) Monk et al., 2002; Berger, 2003).

Brown and Markman (1996) present perhaps the best review of this subject to date. Regarding chemosensitivity assays, the authors state accurately that, "the relevant outcome is whether the assay-directed chemotherapy regimens actually result in improved survival of the patient." No assay-directed chemosensitivity study has demonstrated significant improvement in patient survival compared with empiric therapy. Correlations between in vitro sensitivity and clinical response are fair at best. A meta-analysis of chemosensitivity assays revealed an overall positive predictive accuracy of 69% (Freuhauf and Bosanquet, 1993; Freuhauf, 2002). This percentage is lower than is ideal for clinical practice for selecting therapy, especially in diseases for which standard and effective chemotherapy protocols exist.

In chemosensitivity testing assays, host elements can be found that do not favor accurate in vitro predictions of clinical response in the following ways. (1) Drug delivery is decreased in poorly perfused tumors and in tumor sanctuaries (blood-brain barrier). (2) Altered metabolism in the host's liver may influence activation of pro-drugs (e.g. cyclophosphamide). (3) Individual host sensitivities of normal tissues (bone marrow or stomach mucosa) can lead to life-threatening toxicity, requiring a cutback in drug doses. (4) Local areas of hypoxia or acidosis may inactivate certain drugs. (5) Finally, some

tumors have host-dependent resistance mechanisms that cause high false-positive predictors of in vitro chemosensitivity.

One of the most important factors influencing the accuracy of in vitro predictive assays is the nature of cancer itself. The predictive nature of any laboratory test is a function of both the characteristics of the technology and the patient population to which the test is applied. Chemosensitivity assays were originally modeled after bacterial culture sensitivity tests. However, infectious diseases are highly curable for the most part, and many effective antibiotics are on the market. In contrast, cancers, especially the common solid tumors, are generally chemoresistant.

In practice, the judicious clinician balances the morbidity of therapy against potential benefits in developing a treatment strategy. This is very difficult with diseases that are chemosensitive but for which cure is not possible. Although advanced-stage breast and ovarian cancer are considered incurable, they provide examples of diseases for which dose-intensive therapy has led to significant improvements in patient response, disease-free interval, and overall survival. (DeVita, 1993). The difficulty lies in identifying which patients will benefit from aggressive chemotherapy and which will not. An individual patient is selectively resistant to some agents, while retaining responsiveness to others. Drug resistance is rarely an all-or-none situation. The next section focuses on extreme drug resistance testing by Oncotech Inc. used by the Yale Cancer Center Melanoma Unit to test malignant tumors removed by the surgeon to assay drug resistance. The discussion will begin with a discussion of drug resistance assays to include specifics about the testing method and then resume with a discussion about the advantages and disadvantages of in vitro drug resistance assays in current understanding

with regard to malignant melanoma. The section will include two tables; one that provides an overview of the chemosensitivity and resistance assays represented in a technological assessment and the other that summarize the studies which have been conducted to determine clinical utility of both chemosensitivity and resistance assays.

Drug Resistance Assays

Many retrospective and prospective studies have shown that drug resistance can be determined accurately by in vitro drug-response assays. Unfortunately, reliable drug sensitivity assays for most solid tumors await better therapeutic agents that can accomplish more than palliation. This fact notwithstanding, knowledge of the drug-resistance status of a given tumor can make a significant impact on decision making and treatment planning and is a test result easily understood by the patient and the oncologist. The premise of drug-resistance testing is that identification of inactive agents can contribute to good medical practice by allowing the clinician, surgeon or oncologist to identify agents that will do more harm than good.

Cree et al. (1999) demonstrated considerable heterogeneity of chemosensitivity in metastatic cutaneous melanoma cells using an ex vivo ATP chemosensitivity assay. Tumor cells were exposed to combinations of drugs. Some tumors responded well to one agent or combination, while others showed no response to these drugs commonly used in practice, and instead responded to one of the alternatives. Occasionally highly resistant tumors showed no response to any of the single agents or to combinations of them. The degree of heterogeneity observed suggests that chemosensitivity testing could be used to select patients who might benefit from specific chemotherapeutic agents alone or in combination. This provides the rationale for future randomized control trials of (drug

resistance assay testing) directed chemotherapy versus physician's choice to determine whether assay-directed chemotherapy can improve patient response and survival (Cree et al., 1999).

In vitro extreme drug resistance testing identifies drugs that do not affect tumor tissue within the laboratory setting. The appeal of this can be seen by considering survival statistics associated with malignant metastatic melanoma. Eight months is the mean survival statistic reported for metastatic melanoma (Chapman et al., 2002; Prignano et al., 2002) suggesting that determining in vivo drug effectiveness is difficult at best. Empiric drug response determination in vivo for resistance and sensitivity requires generally at least two months for anti-cancer agents to gauge patient response (Prignano et al., 2002). The determination is made often via experimenting with alternative drugs in a serial manner followed by serial clinical responses. This process represents challenge for the treating physician. This becomes particularly salient when factoring agents that may be clinically ineffective or those that produce toxic side effects or morbidity. As the treating physician attempts to unravel both systemic human response and tumor response, the disease is likely to continue unless the agent administered overcomes the resistance often exhibited in aggressive tumor progression.

ONCOTECH LABORATORY INCORPORATED

Commercial Laboratory Testing of Extreme Drug Resistance

There are a variety of commercial vendors who conduct chemoresponsiveness assays. In the data collected for this study, all of the patient specimens were sent to Oncotech Laboratory Incorporated for analysis. To give some sense of the overall market

in which Oncotech operates, a partial listing of firms is provided. In addition to Oncotech, at least two of the other firms, Impath Incorporated and the Weisenthal Cancer Group provide EDR assays.

Commercial Venues for in vitro Chemoresponsiveness Assays

Rational Therapeutics, Inc
    Offer the ex-vivo apoptotic assay
Oncotech, Inc
    Offer an EDR assay and a DiSC assay
Impath, Inc
    Provide a test similar to the DiSC assay: the ChemoFX assay
Bath Cancer Research Unit (Bath England)
    Provide DiSC assay
Anticancer, Inc
    Patient-based significance of this company is unclear
    Produced GFP-tagged tumors and implant them into mice to visualize metastasis in vivo
NuOncology Labs, Inc
    Provide a cell proliferation assay: an adhesive tumor cell culture system, based on comparing monolayer growth of cells over a proprietary "cell adhesive matrix"
Carl-von-Hess Hospital, Hammelburg, Germany
    Provides a variety of specialized services to patients through its Department of Surgical Oncology (regional chemotherapy, hyperthermia, and phototherapy), in addition to targeted therapy through chemosensitivity assays (ATP-CSA)
Weisenthal Cancer Group
    Provides a variety of cell culture drug resistance tests
Human Tumor Cloning Laboratory, University of Arizona

Oncotech Laboratory Incorporated is located in southern California and has been in service for nearly 20 years. Oncotech in many respects operates much as a research institute would. They oversee an array of research projects and conduct thousands of EDR tests annually. Oncologists and surgeons in the United States and Europe have been using these results to augment or direct the treatment of cancer for their patients for a variety of malignancies.

Oncotech is an innovative company dedicated to the discovery and development of new diagnostic and pharmaceutical products. Oncotech was founded in 1985 and to date, the Oncotech Clinical Laboratory Division has performed oncology services for 100,000 cancer patients from over 1,000 hospitals throughout the nation and Europe since

the company was started in 1990 (Kern and Weisenthal, 1990; Mehta et al., 2001; Holloway et al., 2002; Oncotech, 1999-2004).

The expansion of their facility in the fall of 2001 expanded their relationships with pharmaceutical and in vitro diagnostic companies with their capabilities and technology allowing for rapid advancement in the complex field of proteomics, genomics, and bioinformatics. According to an article released 10/15/03, the expansion of their new molecular genetics research center doubles Oncotech's prior operational capacity. Significant growth has been realized in the number of new hospitals (20% increase) and physicians (25% increases) that began using Oncotech's services in that year. In addition, Oncotech's pharmaceutical services business has grown to include relationships with over 15 companies (Kern and Weisenthal, 1990; Mehta et al., 2001; Holloway et al., 2002; Oncotech Laboratory Incorporated, 2001-2004).

Oncotech performs testing with the following oncologic diseases: brain cancer, breast cancer, cervical cancer, colorectal cancer, leukemia, ovarian cancer, pancreatic cancer, prostate cancer, and more recently melanoma cancer. Oncotech technology includes Extreme Drug Resistance (EDR) Assay, Differential Staining Cytotoxicity (DiSC) Assay, comprehensive Immunohistochemistry and pathology consultation services, advanced flow cytometry and cell sorting technologies, a full array of molecular, biological, and biomechanical techniques, translational genomic and proteomic analysis, and low trauma fine needle aspiration (Kern and Weisenthal, 1990; Mehta et al., 2001; Holloway et al., 2002; Oncotech, 1999-2004).

Oncotech's computer database contains information on more than 80,000 human tumors, including the results of drug resistance testing, prognostic and predictive marker

assays, and pathology testing. Banks of human cancer tumor blocks and sections are maintained by Oncotech, as are frozen cancer tumors and proprietary tumor cell lines (Oncotech, 1999-2004).

The company works within all phases of the advanced drug development process. This includes pre-clinical and clinical studies listed as drug candidate and compound validation through in vitro and ex vivo sample analysis, drug target identification through translational genomic and proteomic analysis of phenotypic isolates, in vitro and ex vivo indication-specific drug efficacy characterization, molecular and functional tumor profiling relative to drug activity, epidemiologic characterization of drug targets within various cancer types, comparison of tumor-specific drug effects relative to that of conventional agents, pre-screening of patients who meet target criteria to optimize response rates for clinical trials, monitoring of patient response through surrogate efficacy assay development and performance, development and marketing of drug-specific theragnostic assays clinically to increase market penetration of approved drugs, drug indication expansion through drug synergy studies, treatment failure analysis and tumor marker surveys (Kern and Weisenthal, 1990; Mehta et al., 2001; Holloway et al., 2002; Oncotech, 2001).

The company conducts animal studies in parallel if requested, with the pre-clinical in vitro human tumor studies such as human xenografts analysis, minimally invasive in vivo monitoring of drug activity within animal tumors using fine needle aspiration devices, assessment of pharmacokinetic and pharmacodynamic activity in vivo, and surrogate efficacy marker validation and apoptotic response (Kern and

Weisenthal, 1990; Mehta et al., 2001; Holloway et al., 2002; Oncotech 1999-2004; Freuhauf et al., 2004).

Oncotech's EDR Assay was developed for testing in vitro drug responses in solid tumor cancers and is the only solid tumor assay capable of identifying extreme drug resistance with over 99 % accuracy (Fruehauf and Bosanquet, 1993; Chu and De Vita, 2001; Freuhauf, 2002). Validation of Oncotech's EDR assay technology has been documented in 450 clinical correlations obtained over an eight year period. Results of this study were published in the *Journal of the National Cancer Institute* (Kern and Weisenthal, 1990; Oncotech, 2001-2003).

The practical utility of in vitro testing has been enhanced by the development of the third generation assay technique used at Oncotech. Older clonogenic systems yielded results in two to three weeks with 50% success rates. Newer EDR techniques like the one developed and used at Oncotech have shortened assay time to less than one week and improved the evaluability rate to 85% which is similar to the evaluability rate of 91% in the Mehta study Mehta et al., 2001).

Oncotech EDR Test Results

The test results from Oncotech Laboratory consist primarily of a categorization of level of drug resistance of the tumor sample to the drug used in the EDR in vitro assay. For melanoma, the tumor samples are tested for extreme drug resistance. The level of resistance is reported as either low drug resistance, intermediate drug resistance, or extreme drug resistance.

Extreme Drug Resistance (EDR) indicates that tumor cell growth was virtually unaffected by the high chemotherapeutic agent exposure. Data published in Kern and

Weisenthal (1990) show that patients have less than a 1 percent chance of responding to EDR agents. EDR is defined as one standard deviation more resistant than the median result for comparison.

Intermediate Drug Resistance (IDR) indicates moderate tumor growth. In Kern and Weisenthal (1990) patients treated with agents in the IDR category had response rates that were about half of the rates reported in the medical literature. The IDR is a result more resistant than the median but less resistant than EDR.

Low Drug Resistance (LDR) indicates that tumor cell proliferation was inhibited by the tested agent and that tumor cells demonstrated less than median growth. Patients treated with agents in the LDR category had response rates that were approximately 1 and one-half to 2-fold greater than the literature reported rates (Kern and Weisenthal 1990). The LDR is a result less resistant than the median.

The Literature Response Rate is determined from an extensive review of clinical trials in which each drug was administered as single agent therapy to specific tumor type. The Assay Predicted Response Probability was derived from an algorithm involving in vitro tumor cell proliferation, literature response rate, patient treatment status, and a comparison with a growing database of over 80,000 in vitro assays, in accordance with the Bayesian mathematical model as discussed previously in this chapter (Kern and Weisenthal, 1990; Oncotech, 2001-2003).

The drug resistance testing protocol biases assay reliability toward accurate detection of drug resistance. In correlating in vitro drug resistance with clinical response, the prediction of resistance may be considered is more robust than the prediction of

sensitivity in the ability of in vitro systems to parallel relevant in vivo pharmacodynamics.

The following is an overview of the chemosensitivity and resistance assays represented in a technology assessment (Schrag et al., 2004).

Table 10 Chemosensitivity and resistance assays technology assessment (Schrag et al., 2004)

Assay Name	Target Tumor Types*	Assay Description
SRCA (Maenapp et al. 1995)	Epithelial ovarian cancers	Human tumor specimens are cultured in the sub renal capsule of mice. Tumor growth in mice is measured following treatment with various drugs of saline to determine drug sensitivity.
HTCA and CCS (Von Hoff et al, 1983; Von Hoff et al., 1990)	Multiple tumor types	Single cell suspensions prepared from patient's tumors are cultured in vitro for several weeks. The colony-forming efficiency of these cells in the presence and absence of various drugs is evaluated to determine drug sensitivity.
DiSC (Gazdar et al., 1990; Shaw et al., 1996; Von Hoff et al., 1983, Wilbur et al., 1992)	Lung cancer (small and non-small cell)	Tumor cells are cultured in vitro in the presence/absence of three concentrations of drug. After a 6-day incubation, differential dye staining is used to identify viable cells and determine drug sensitivity.
MTT (Kurbach et al., 1998)	Breast cancer	Tumor cell suspensions are cultured with various chemotherapy agents for 4 days. MTT is then added; because it reduces intracellularity to a blue dye the intensity of uptake yields an estimate of the number of viable cells to determine drug sensitivity.
ATP (Kurbacher et al., 1998)	Epithelial ovarian cancer	Tumor cell suspensions are cultured in the presence/absence of test drugs and then cells are lysed. The amount of ATP-a reflection of the number of viable cells-is measured by adding luciferin (the same compound which causes fireflies to glow). Low ATP concentration manifests as low luminescence to identify efficacious test drugs.
EDR assay (Kern et al., 1985; Kern and Weisenthal, 1990)	Epithelial ovarian cancer	After successful culture, tumor cells obtained from fresh biopsy specimens are labeled with titrated thymidine. The level of uptake is tracked after exposure to chemotherapy concentrations that approximate the peak level achieved clinically. Extreme resistance is identified when thymidine incorporation is inhibited in the presence of the drug by less than one standard deviation of the median cell inhibition measured for several hundred reference tumor samples.

The following is a table summarizing the studies which evaluate the clinical utility of chemotherapy sensitivity and resistance assays (Schrag et al., 2004).

Table 11 Clinical utility of sensitivity and resistance assays (Schrag et al., 2004)

Study and Assay	Design	Proportion of Patients With Assessable Assays*	Summary of Main Findings + (tumor response rates reported)
HTCA (Von Hoff, 1983)	Comparison of the rate of complete & partial responses to chemotherapy for patients with metastatic cancer for 3 non-randomly assigned groups: I) Successful tumor cell culture & assay-guided tx II) Cells not successfully cultured, empiric tx III) Successful cell culture but empiric tx delivered because pt. refused assay-guided tx or contraindication to assay recommended agent.	64% (303 of 470) pt) assessable; the unit of analysis was the number of assay trials and not the multiple assays performed per pt.	Assay-guided (group I): 62 or 246; 25% Empiric (group II): 39 of 256' 14% Empiric (group III): 11 of 102; 11% Study weaknesses: the non-random treatment assignment, cells could not be assayed in many circumstances because they did not grow in culture.
HTCA (von Hoff, 1991)	Patients with metastatic tumors whose HTCA assays identified a chemoresponsive agent were treated with that drug. In vivo complete and partial responses to treatment were measured: Group I: 18 patients received HTCA-guided therapy; These patients were derived from cohort of 75 patients with assessable assays, 31 of 75 of whom had sensitive assays.	45% (75 of 168 pts) assessable	Assay-guided (group I): 5 of 18; 28% Empiric (group II): 10 of 90; 11% Study weakness: the non-random treatment assignment & the inclusion of pts with assay-predicted unresponsiveness tumor in the comparison group, which comprises interpretability. The analysis did not directly compare assay vs. non-assay-guided therapy.
CCS (Von Hoff, 1990)	Randomized controlled trial: Group I: 68 pts received assay-guided therapy; 19 pts assessable for response in assay-guided group. Group II: 65 pts received clinician's choice of therapy; 36 pts were assessable for response.	71% (48 of 68 pts) assessable	Assay-guided (group I): 4 of 19; 21% partial response Empiric (group II): 1 of 36; 3 % partial response There was a higher response rate for drug selection based on CCS than by physician choice, but no survival difference. The strength of this study is the randomization; a weakness is that only a small proportion of pts actually received tx according to randomization, as well as a lack of complete response data from either group.
DiSC (Gazdar, 1990)	Comparison of responses to second-line chemotherapy for small-cell lung cancer for 2 randomly-assigned groups: Group I: Assay-guided. Successful DiSC assay (n=26); assay-guided therapy (n=16). Group 2: Empiric.	33% (26 of 79 pts) assessable	Assay-guided (group I): 4 of 16; 25% Empiric (group 2): 3 of 43; 7% Study weakness: not all pts with successful DiSC assay received assay-guided tx, and

	Unsuccessful assay (n=53); empiric regimen of vincristine, doxorubicin, and cyclophosphamide (n=43).		a high proportion of pts lacking assay results received assigned regimen.
DiSC (Wilbur, 1992)	No randomization to assay-guided tx. The DiSC assay was used to measure cell kill in the tumor cell population among a nonconsecutively ascertained prospective cohort of 45 pts with advanced NSCLC. Treatment with the 3 best drugs selected by the assay was administered to 25 of the 35 pts who had a successful assay.	78% (35 of 45 pts) assessable	Assay-guided: 9 pf 25; 36% Empiric not reported Response rate among 12 pts for whom the assay indicated that tumor cells were sensitive to chemotherapy was higher (6 of 12; 50%) than response rates among 13 pts for whom assay indicated resistance (3 of 13; 23%). Results were similar irrespective of the cutoff point used to define sensitive/resistant.
Modified version of DiSC termed Drug Sensitivity Testing (Shaw, 1993, 1996)	No randomization to assay-guided tx.	Cohort 1: SCLS 29% (33 of 115 pts) assessable	SCLS (cohort 1)
MTT (Xu, 1999)	Non-randomized prospective study of 156 women with metastatic breast cancer	88% (73 of 83 pts) assessable	Assay-guided: 56 of 73; 77% Empiric: 32 of 83; 39% No difference in either the median response or median survival
DiSC (Cortazar, 1997)	No randomization to assay-guided tx A prospective cohort of extensive stage SCLC had DiSC performed on cell lines established after pre-tx biopsy & after initial tx with VP-16/CDDP. The DiSC was used to guide the second 12 weeks of chemotherapy similar to the design by Shaw. Assay-guided tx was administered when DiSC was successful & when DiSC results were unavailable, empiric tx with VAC was given.	56% (10 of 18 pts) assessable	Assay-guided: 8 or 8; 100% Empiric: 44 of 44; 100% Superb response rates in both groups
APT (Kurbacher, 1998)	No randomization to assay-guided tx A prospective cohort of women with recurrent ovarian cancer had assay-guided tx if the ATP was successfully performed & the primary physician agreed to abide by the tx assignment (or alternatively, with empiric tx).	93% (29 of 31 pts) assessable	Assay-guided: 16 of 25; 64% Empiric: 11 of 30; 37% There were major differences in the tx regimens selected between 2 groups. Whereas 23 of 25 pts receiving assay-guided tx had combination regimens, 21 of 3o receiving empiric tx had combination regimens. 12 pts in the assay-guided arm received paclitaxel on a protocol, whereas none of the empiric tx group received a taxane.
EDR (Lozzi, 2003)	No randomization to assay-guided tx Retrospective analysis of 50 women with recurrent ovarian cancer treated with assay guidance & 50 women with recurrent ovarian cancer	100% (50 of) 50 pts assessable	Assay-guided: 28 of 50; 56% Empiric therapy: 14 of 50; 28% Platinum-resistant or – sensitive disease was defined

	treated empirically.		based on weather interval from last treatment to progression was more or less than 6 months. Difference between assay and empiric group was greatest for the subset with platinum-sensitive disease. Although there was a statistically significant difference in survival, the authors acknowledged that the study was not designed to determine a statistical difference between assay-guided & empiric tx groups. They note that the survival analysis was conducted for exploratory, hypothesis-generating purpose only.
SRCA (Maenpaa, 1995)	Prospective randomized trail: Group I: 98 pts with epithelial ovarian cancer (stage II-IV) assigned to assay-guided tx (various combination regimens). Group II: 98 pts with epithelial ovarian cancer (stage II-IV) assigned to empiric tx (CAP).	70% (60 of 98 pts) assessable	Assay-guided: 39 of 63; 62% Empiric tx: 41 of 69%; 59% No survival benefit observed. 24 of 63 (38%) pts in assay-guided group received empiric-group tx of CAP. High number of dropouts.

Abbreviations: HTCA, human tumor cloning assay; CCS, capillary cloning system; DiSC, different staining cytotoxicity NSCLC, non-small-cell ling cancer; SCLC, small-cell ling cancer, VP16, etoposide; CDDP, cisplatin; MTT, methylthiaolyl-diphenly-tetrazolium bromide; VAC, vincristine, doxorubicin, and cyclophosphamide; ATP, adenosine triphosphate bioluminescence; EDR, extreme drug resistance assay; SRCA, subrenal capsule assay; CAP, cyclophosphamide, doxorubicin, and cisplatin

*Sample size refers to the number of assessable patients. For the most chemosensitivity and resistance assays, the number of tumor specimens for which assays were initiated greatly exceeded the number ultimately evaluated because of difficulty isolating and preparing tumor cells for in vitro analysis.

+ Tumor response rates reported in the table were assessed based on complete plus partial responses.

Chemoresistant assays are based on the same principles as the chemosensitive assay. However, these assays focus on negative predictive accuracy (NPV) (i.e. the ability of the assay to identify ineffective agents) rather than positive predictive accuracy (PPV) (i.e. ability of the assay to identify agents capable of inducing a clinical response); therefore sensitivity and resistance are not the same phenomenon seen from two different points of view.

Results of the EDR Assay are used to determine the probability of non-response (extreme drug resistance) by the tumor to the selected chemotherapeutic agent. To accomplish this, the laboratory uses thymidine incorporation to calculate the proliferation of tumor cells after they have been plated in a 3D agarose media and exposed to a variety of chemotherapy agents.

In general, most solid tumors such as lung, ovarian, breast, and melanoma, may be treated with different combinations of chemotherapeutic agents. When EDR is present, it is usually possible to eliminate the resistant drug(s) and still formulate a standard treatment regimen. Thus, the greatest utility of the EDR Assay is found in those tumor types where more than one standard chemotherapy option exists.

The EDR assays are not to be confused with the traditional (clonogenic) chemosensitivity assays. The success of this assay results from extended exposure of the patient tumor cells to chemotherapy agents which approximate the peak plasma levels attained after conventional IV administration. If a patient's cells proliferate after extended exposure to plasma levels of chemotherapy agents, then it can be accurately predicted that these cell will also demonstrate resistance to normal exposures in vivo.

Kern and Weisenthal introduced the EDR concept in 1990. The assay developed by Kern and Weisenthal uses a longer drug exposure than the clonogenic assay, biasing the capability toward detection of drug resistance. Therefore, the method was labeled the "Extreme Drug Resistance Assay" (Kern and Weisenthal, 1990).

The drug-resistance assay was calibrated to produce extremely high specificity for drug resistance. A large pool of samples was used to establish thresholds for categorizing

drug resistance. The rate of cell growth relative to those external standards is then used to classify drug resistance.

At the lower cutoff value (one standard deviation below the median), the assay was 99% specific in identifying nonresponders. These samples are considered to exhibit extreme drug resistance. The patients with assay values below this cutoff had an actual response rate of less than one percent. Especially notable was the fact that patients with drug-resistant tumors could be accurately identified in otherwise highly responsive patient cohorts. Tumors exhibiting percent cell inhibition (PCI) values above the median were placed into the low drug resistance category: tumors with PCI values between the median and one standard deviation below the median were placed into the intermediate drug resistance category. The investigators concluded that the assays' high specificity for drug resistance could be primarily attributed to (a) the high drug concentration used, (b) avoidance of technical artifacts through attention to technical detail, and (c) use of appropriate positive and negative controls.

When the subset of patients with extreme drug resistance (EDR) was excluded from the analysis, the response rate for the remaining patients was higher than expected. The response rate increased as the percent inhibition of cell growth in the assay increased. Prediction of response probability in the patients without EDR was not only a function of the assay result but also a function of the pretest probability of response. When the pretest probability was low, the assay identified patients with response rates higher than expected, but these predicted response rates were lower in an absolute sense than those for patients with higher pretest response probabilities (e.g. breast cancer or ovarian cancer).

Operationalizing the EDR technique requires surgical excision of a tumor sample. The fresh tissue is sent to a laboratory for processing. Typical processes for one such laboratory (Oncotech Laboratory Incorporated) for lymphoma and malignant effusions are listed here. The Assay Specimen Preparations Guidelines (Kern and Weisenthal, 1990; Mehta et al., 2001; Holloway et al., 2002; Oncotech, 1999-2004) are as follows:

For solid tumor

Obtain fresh biopsy specimen. Do not mince, fix, or freeze specimen.
Rinse specimen in sterile saline or lactated Ringer's solution.
Immediately place sample into the inner specimen transport vial which contains Oncotech transport media.
In the absence of transport media, use sterile lactate Ringer's solution or RPMI 1640.
Secure inner and outer specimen vials tightly.
Place specimen vial assembly into center of the Oncotech box. Place ice pack on top of vial assembly.
Enclose completed requisition from.
Place closed box into Federal Express Diagnostic Pack for shipment.
Call listed phone number for specimen pick-up.
Refrigerate transport vial until use.
Freeze Oncotech transport box at least 24 hours before use.
Patients must not have had chemotherapy or radiation therapy within 3 weeks of specimen collection.

For malignant effusions:

Collect 25-1000ml of fluid in a sterile evacuation bottle or polyethylene bag. Do not use Pleur-Evac system containers.
Add 3 drops of heparin per l ml of fluid. Example: For stock solutions of heparin containing 10,000 units per ml, add 0.3ml of heparin solution per 100ml of malignant fluid.
Retain a portion of the fluid for cytology.
Refrigerate the specimen until shipment.
Use an Oncotech leak proof, protective shipping container.

The EDR Methodology at Oncotech Inc.

The fresh viable tumor tissue is minced and reductive enzymes are applied to disaggregate the tumor cells. The tumor cells are plated in soft agar which favors tumor cell proliferation relative to stromal cells. The cells are exposed to tumor type-specific antineoplastic agents for five days in a carefully controlled environment. Due to the reduced rate of drug metabolism, in vitro tumor exposure is greater than in vivo. Tritiated thymidine is introduced during the last two days of culture as a measure of cell

proliferation. Treated cells are compared to untreated controls. If malignant cells proliferate in vitro under such extreme chemotherapeutic exposure conditions, then in vivo exposures will be ineffective, with a probability greater than 99% (Kern and Weisenthal, 1990; Mehta et al., 2001; Holloway et al., 2002; Oncotech, 1999-2004).

The advantages and disadvantages of a given endpoint are related in part to their ease of use, reproducibility, precision, and success rate. Assay endpoints are generally related to measures of cell proliferation, metabolism, or survival. The HTCA measures the capability of single malignant cells to divide and form colonies in or on agar-based matrix. After short-term drug exposure, single cells are plated in or on an agar matrix. Cells that have been killed or have undergone damage causing cell cycle fail to form colonies. Colony counts after a two-three week period is the end point determined in the HTCA. Differential colony formation between untreated controls and treated cells measures drug activity. Agar, the growth substrate, mimics a suspension environment and suppresses the proliferation of nontransformed cells (Puck and Marcus, 1955; Hamburger and Salmon, 1977). Agar-based culture systems, such as used in the EDR assay, or polypropylene plates employed in the DiSC and ATP assay systems, suppress cellular adherence to a growth surface.

Fibroblasts, mesothelial cells and other stromal cells can proliferate in adherence-based culture systems, adding a non-cancer cell-specific growth signal or component to the endpoint. In vitro drug-response assay results are adversely affected by proliferation of non-malignant cells that add 'noise' to the cancer cell growth signal (Campling et al., 1991). The growth signal of disaggregated cells obtained from tumor biopsies grown in non-adherent culture conditions is therefore relatively more cancer cell specific than

growth in adherent culture systems. The use of low serum-containing media is another technique employed to suppress non-transformed cell proliferation. The use of agar with low serum helps ensure that assay endpoints determined after several days of tissue culture measure cancer cell proliferation or metabolism with minimal contributions from normal cellular components present in each tumor biopsy specimen.

Host factors may favor the accurate predictions of clinical drug resistance. If cancer cells are found to survive high exposures of drugs in the laboratory, host factors may not render the cells responsive in vivo. However there are possible effects of the drugs on the immune system or vasculature as is the subject of the research of others as mentioned in this paper. Questions of combination testing multiple drug synergies are relevant for chemosensitivity assays. In the EDR assay the drugs are generally tested as single agents. This may be seen as an advantage to extreme drug resistance testing. If combinations are tested, only one drug out of two or three in the combination need be active for the combination to show sensitivity, and drug resistance can be masked. Synergy may play a role in some drug combinations, such as has been observed with 5-fluorouracil and methotrexate and with cisplatin and etoposide (Bertino et al., 1983; Zupi et al., 1985). However, it seems that synergy is primarily found in drugs that have relative significant activity against cancer cells. When tumor cells show extreme drug resistance to a particular anticancer drug, the modulating effects of other agents may be nullified (Sondak et al., 1988a; 1988b).

If an accurate and consistent strategy were available to oncologists to use customized information to make chemotherapy recommendations that were tailored specifically to a patient's tumor characteristics, it may enhance the decision-making

process. The approach has enormous intuitive appeal and may be more logical to both patients and physicians than the empiric approach, whereby all patients with similar tumor type are treated according to a standardized regimen. However, obstacles will remain before CSRSAs are integrated into general clinical care (Schrag, et al., 2005). To date, the available literature on CSRAs does not support use of this technology outside of a clinical research lab (Schrag et al., 2005). Next follows studies which have incorporated EDR in vitro drug testing that have shown promise to day in breast, ovarian and melanoma malignancies.

IN VITRO ASSAY TESTING IN CANCER STUDIES

Efficacy of In Vitro testing for Breast and Ovarian Cancer

While this paper is primarily directed at EDR testing in the case of melanoma, the concept has been applied in the context of other cancers. The analytical approaches towards the understanding of drug resistance, predictive accuracy or value, correlations with tests for biomarkers, and correspondence with clinical outcomes in other cancers are instructive in understanding which issues have and have not been addressed in the case of melanoma. Here, the development of the EDR literature with respect to other cancers is discussed as it informs the discussion of melanoma research.

Clinical trial-based identification of new chemotherapy agents with significant disease specific activity has been a cornerstone of modern oncology, providing statistical validation of their safety and activity. Perhaps of greatest importance, the impact of new treatment modalities on patient survival can be compared with previously proven

treatment regimens. The clinical utility of agents developed through this process has led to significantly improved outcomes for women with advanced stage breast and ovarian cancer. Despite these advances, disease progression and patient death are still major problems that largely result from intrinsic and acquired drug resistance. Although current chemotherapy regimens produce clinical response rates for women with breast cancer of 60 to 70%, five-year survival rates for these women remain below 50%, and cures are rare (Greenlee et al., 2001). Fruehauf and Alberts (2003) echo this disappointing statistic citing large randomized trials which examined the clinical activity of chemotherapy regimens against both breast and ovarian cancer. Several agents are available that can prolong lives, and the authors' state that the clinical activity of these chemotherapy regimens is initially high, with 70% of patients responding.

Unfortunately the authors point out their benefit in second-line selection is often limited, with less than 30% of patients showing significant disease response. Thus some 70% of patients may undergo effective treatment during the course of their disease but develop a drug resistance which inhibits the effectiveness of treatment under relapse.

The selection of chemotherapy for women with breast or ovarian carcinoma has traditionally been based on results from phase III comparative trials that define the most active single and combined drug therapies (Fruehauf, 2002). This approach has lead to a significant prolongation of the lives of these patients. Unfortunately, few patients with advanced stage IV disease are cured using the currently available regimens. In order to improve the selection process for individual patients, various types of in vitro tests that assess the activity of standard drugs on a patient's tumor have been developed over the past five decades. As with bacterial culture and sensitivity tests, significant predictive

correlations between in vitro drug-response assays and cancer patient response and survival have been demonstrated.

The clinical utility of in vitro drug-response assays has been evaluated in trials (Alberts et al., 1980; Kern and Weisenthal, 1990; Blackman et al., 1994; Kochli et al., 1994; Elledge et al., 1995; Csoka et al., 1997, Taylor et al., 1998, Xu et al., 1998; Konecky et al., 2000; Freuhauf, 2002). The trials examined the relationship between drug action on a given patient's tumor in vitro and that patient's clinical response to that drug. A recent study addressed the predictive accuracy or value of various in vitro drug response technologies in a sample of 220 breast cancer cases and 284 ovarian cancer cases. They defined negative predictive accuracy or value as the reliability of the assay in identifying ineffective agents, while the positive predictive accuracy or value measures clinical responses defined as a reduction in measurable tumor size of at least 50%. The negative predictive value for breast cancer ranged from 86 to 100%, while the positive predictive value ranged from 58 to 9%. While these ranges are rather broad, it appears that the NPVs were generally higher than the PPVs, suggesting that these technologies were better at identifying ineffective agents. This has been a generally accepted axiom for years based on the clear differences between in vitro models and in vivo pharmacodynamics (Freuhauf, 2002).

While it is clear that in vitro drug response assays effectively discriminate between clinically inactive and active agents, this does not necessarily translate to an accurate prediction of patient survival. Various clinical trials have identified agents capable of causing short-term responses without translating into a survival benefit. Clinical validation of in vitro drug-resistance assays requires that they predict poorer

survival for patients treated with agents when their tumors are drug resistant in vitro and improved survival for patients treated with agents found to exhibit sensitivity in vitro.

In Vitro Assay Testing in Breast Cancer

Statistically breast cancer is prevalent on a global scale. It is one of the cancers that in vitro testing has been utilized. Its utilization and trial results will be discussed in this section. Breast cancer is by far the most frequent cancer of women (23% of all cancers) in the world, with an estimated 1.15 million new cases in 2002, ranking second overall when both sexes are considered together (Parkin et al., 2005). More than half the cases are in industrialized countries. Incidence rates are high in most of the developed areas of the world in part because of the presence of screening programs that detect early invasive cancers. Incidence rates are more modest in Eastern Europe, South America, Southern Africa, and Western Asia, but it is still the most common cancer of women in these geographic regions (Parkin et al., 2005). The prognosis from breast cancer is generally rather good. As a result, breast cancer ranks among the fifth cause of death from cancer overall, although still the leading cause of cancer mortality in women (Parkin et al., 2005).

The very favorable survival in the more affluent developed countries and poor survival in some of the least affluent countries results in the differences in mortality rates worldwide being much less marked than for incidence. The estimated mortality rates in Africa, for example, are not greatly inferior to those in Europe. Because of its high incidence and relatively good prognosis, breast cancer is the most prevalent cancer in the world today (Parkin et al., 2005); there are an estimated 4.4 million women alive who have had breast cancer diagnosed within the last five years (compared with just 1.4

million survivors-male or female from lung cancer). It has been estimated that 1.5% of the U.S. female population are survivors of breast cancer (Hewitt, Breen and Devesa, 1999; Parkin et al., 2005)

The selection of chemotherapy for women with breast or ovarian cancer has been traditionally based on results form phase III comparative trials that define the most active drugs and drug combinations (Freuhauf, 2002). That approach has led to a significant prolongation of the lives of these patients. Unfortunately, few patients with advanced stage IV disease are cured using the currently available regimens. In order to improve the selection process for individual patients, various types of in vitro tests have been tried over the last five decades.

Multi-agent chemotherapy continues as an important component of treatment for invasive breast cancers greater than 1cm in size. Combination chemotherapy exploits the Goldie-Coldman hypothesis by targeting the heterogeneous malignant clones within each patient. This strategy led to the development and clinical validation of various standard combination chemotherapy regimens comprised of non-cross-resistant agents. The inability to demonstrate a clear superiority of one regimen over another, or the superiority of high dose combination regimens over standard dose chemotherapy, suggests that a plateau in benefit may have been reached using the current non-targeted, empirical approach to treatment selection.

The empirical use of one of the standard regimens does not routinely take into account that patient's unique tumor biology. The initial proof of the principle that targeted therapy could be a useful strategy stemmed from observations that tamoxifen

treatment could significantly improve survival in patients with estrogen receptor (ER) positive tumors (Early Breast Cancer Trialists' Collaborative Group, 1998).

This observation along with a similar study of herceptin and paclitaxel in clinical trials has validated the concept of individual patterns of drug specific resistance. Some patients failed to respond to single agent paclitaxel, yet subsequently responded to non-cross resistant doxorubicin on cross over, or visa versa (Sledge et al., 1997). These observations suggest that the ability to identify individual patterns of resistance prior to initiating chemotherapy might have substantial clinical impact and can potentially avoid toxicity, lost time, and costs associated with ineffective treatment (Orr et al., 1999).

Mehta et al. (2001) conducted an impressive study testing whether in vitro EDR assay results for patients with breast carcinoma were associated with clinical outcome after chemotherapy. This study also examined the relationship between in vitro EDR assay results, and progression-free and overall survival. EDR assay results were obtained for a serial cohort of 103 cases prior to first line chemotherapy. The treating oncologist was blinded to EDR results and lab personnel were blinded to clinical characteristics. Between October 1990 and March 1996 tissue samples from 187 serial patients with newly diagnosed invasive breast cancer for which tumor tissue could be obtained were sent from a single NSABP institution (to Oncotech Laboratory Incorporated) for in vitro EDR testing. The drugs, either cyclophosphamide, methotrexate, 5-fluorouracil (CMF) or adriamycin, cyclophosphamide (AC), that were added to malignant cells for testing were at doses that approximated their in vivo peak plasma concentrations.

Patients in this study were stratified according to the sum of the EDR scores for the agents they received. Patients who showed in vitro resistance at an intermediate or

extreme level had progression-free survival rates decreased by half in multivariate analysis adjusted for stage and lymph node status. A significant difference in survival was also noted between patients with intermediate to extreme drug resistance. Patients with intermediate or extreme drug resistance demonstrated significantly shorter survival, with five-year survival rates of 45% compared to 81% in patients with low drug resistance. Patients who received agents resistant in vitro had a threefold increased relative risk of death.

In vitro resistance patterns varied among patients. Few patients showed resistance to all drugs tested. This suggests that alternative agents may have been available to choose from for the majority of patients when one specific agent was found to be inactive in vitro. Further there was a significant association between in vitro drug resistance for single agent 5-FU and progression-free and overall survival of patients treated with CMF. This suggests that the clinical activity of 5-FU in the CMF regimen may be a major determinant of outcome for patients.

The outcome for this study demonstrated a clear relationship between the degree of in vitro resistance seen in the EDR assay and clinical outcome. It also demonstrated a significant association between survival and EDR assay results for primary tumor tissues obtained from breast cancer patients prior to chemotherapy. The investigators concluded that EDR testing identified patients with individual patterns of drug resistance prior to therapy. This supports the notion that drug resistance testing can identify a tumor phenotype related to clinical outcome. In this cohort of breast cancer patients treated with chemotherapy, summed EDR scores were significantly associated with time to tumor progression and overall survival (Mehta et al., 2001).

The improved survival demonstrated for patients treated with combinations of low resistance agents is intriguing. In an early 1990 review, 12 of 17 studies were found to demonstrate a statistically significant survival advantage for patients treated with agents to whom they were sensitive in vitro (Freuhauf and Bosanquet, 1993). There are a total of 179 published correlations between assay results and patient treatment. According to Weisenthal (2004), patients treated with assay "sensitive" drugs had a 92% response rate. Patients treated with assay "resistant" drugs had a 7.7% response rate. The overall response rate for the patients in the studies was 66%.

Xu and colleagues (1999) treated 77 breast cancer patients on the basis or MTT-assay directed chemotherapy and compared outcomes with 73 patients treated with "physician's choice" chemotherapy. This study was non-randomized. The response rate of the assay-directed group was 77%, while for the empiric group, 44%. The patients receiving assay-directed therapy actually had less favorable prognostic factors, but showed a response advantage to assay-directed therapy and a trend for survival advantage (one year survivorship for assay-directed therapy was 74% and 67% for empiric therapy, three year survivorship were 25% and 19% respectively, five year, 20.5% and 12.3%, respectively).

Similarly, Gambino et al. (1999) demonstrated high response rates with assay directed therapy in patients with chemotherapy refractory gynecological malignancies. A prospective trial by Kurbacher et al. (2003), demonstrated a high response rate and promising survival outcomes in recurrent ovarian cancer treated with therapy tailored according to their in vitro results.

Sampson et al. (2004) conducted a systematic review of chemosensitivity and chemoresistant assays. These investigators found that while higher response rates for assay-directed therapy may be observed in clinical trial studies, these differences may be attributable to bias or confounding. The investigators found that only modest evidence on survival is available. The investigators go on to say that the overall results available from the studies they evaluated that have been conducted to date, do not establish the relative effectiveness of assay-guided treatments nor empiric treatment and as a result, they suggest randomized trials are needed.

In Vitro Assay Testing in Ovarian Cancer

Epithelial cancer is the most lethal of gynecologic malignancies, and the forth leading cause of cancer death in American women between the ages of 40 and 59. Approximately on woman dies from advanced disease every 45 minutes. In the United States, ovarian cancer causes approximately 14,500 deaths annually (Parkin et al., 2005). Ovarian cancer is the sixth (204,000 cases and 125,000 deaths) most common cancer and the seventh cause of death from cancer in women world-wide (4.0% of cases and 4.2 deaths). The incidence rates are the highest in developed countries, relatively high in South America and slowly increasing in many Western countries and Japan (Parkin et al., 2005). With an incidence of one in 60 approximately 22,220 new cases were to be diagnosed during 2004 (Jemal et al, 2005; Krishnansu et al, 2005).

Cisplatin-based therapy has significantly improved the duration of survival in patients with advanced cancer, its impact on cure is less certain. Although primary ovarian carcinomas initially respond to platinum-based chemotherapy in up to 80% of women with advanced disease, responses typically are incomplete and most such patients

will relapse. Accordingly, despite good initial responses to chemotherapy, 75% of women with stage III and IV disease die of complications associated with disease progression. The five-year survival rate is about 25%. Given the high recurrence rate and poor long-term survival of women with advanced ovarian cancer, there is a strong impetus to investigate new technologies that might permit more effective treatment of women who have recurrence. EDR assays to assist in making treatment decisions for women with ovarian cancer have received considerable attention (Loizzi et al., 2003).

Loizzi et al. (2003) evaluated 100 patients between 1993 and 2002 with recurrent ovarian cancer at first relapse at University of California, Irving and Long Beach Medical Memorial Center. In their retrospective analysis, their results showed an improved response rate and duration of progress-free and overall survival in patients with platinum-sensitive disease who underwent second-line chemotherapy that was guided by EDR assays compared with those who were treated by clinical judgment alone. This suggests there may be a well-defined group for who EDR testing might benefit. Assay-directed therapy did not affect the outcome of women with platinum-resistant recurrent ovarian therapy. The general conclusion about EDR assay utilization as made by Loizzi et al. regarding assistance with treatment decisions in ovarian cancer appears to rest with their ability to exclude certain drugs with very low likelihood (<1%) of clinical activity rather than their ability to reliably predict agents that will show clinical activity. Because of realities in short-term follow-up and limited number of patients in the study, these authors were not able to demonstrate an overall survival benefit using assay-directed therapy (Loizzi et al., 2003).

One recent study of EDR testing in ovarian cancer (Krishnansu et al., 2005) compared at total of 6990 epithelial ovarian cancer specimens submitted to Oncotech from 1990 to 2000. All specimens were obtained from women with advanced primary or recurrent International Federation of Gynecology and Obstetrics (FIGO) surgical stage III or IV disease. The data suggest that assay results at diagnosis may be useful in guiding therapy at relapse, especially when managing a chemical recurrence or one in which tissue is not available for drug resistance testing. Due to a failure to demonstrate any survival benefit, reassessment laparotomy following primary therapy has, for the most part, been relegated to investigational protocols and secondary debulking remains controversial. Thus, the likelihood of submitting tumor for chemosensitivity testing is highest at initial diagnosis since most patients undergo primary debulking and few undergo secondary and tertiary cytoreductive surgeries. The data suggest that the molecular changes that lead to drug resistance may occur early in the carcinogenesis process, perhaps before metastases have been established. According to the authors of the study an analysis of metachronous tissues retrieved from less advanced patients who ultimately relapse following therapy may she additional light onto this paradoxical subject (Krishnansu et al., 2005).

In their study in 1990, Kern et al. evaluated the EDR method testing ovarian cancer. The EDR assay method was tested on 450 patients with various tumor types and with measurable disease. The EDR assay was demonstrated to be 99% accurate at identifying agents that failed to produce clinical response. Of the 46 ovarian cancer patients in that study, no responders were seen in the subset treated with agents falling into the EDR category. While it is expected that patients unlikely to respond to treatment

based on in vitro results would have an inferior outcome, no large study had at that time addressed the relationship between EDR assay results and survival in a uniform group of patients with newly diagnosed ovarian cancer.

Holloway et al. (2002) evaluated in vitro response as assessed by the EDR assay method. The tests in their study were conducted by Oncotech. Surgical biopsies from metastatic sites were obtained during primary debulking surgery and sent for analysis. Both positive and negative controls were performed with each assay. Results were reported as percent cell inhibition (PCI) for the individual drug compared with media-exposed control cultures after subtraction of positive control. EDR assay performance characteristics were determined for cisplatin, carboplatin, 4HC, and paclitaxel, on 11,000 independent cases previously evaluated using the same methods, The individual patient's tumor response to each drug was compared to their calculated PCI, which was compared to the population median and SD determined for that drug. Tumors exhibiting PCI values above the median were placed into the low drug resistance category; tumors with PCI values between the median and 1 SD below the median were placed into the intermediate drug resistance category. Those tumors with PCI values that were more than 1 SD below the median were placed into the extreme drug resistance category. Progression-free survival and overall survival of patients with tumors demonstrating EDR to one or both platinum compounds were compared with that of patients demonstrating intermediate or low drug resistance to platinum compounds.

The EDR assay success rate for malignant specimens submitted by the principle clinical investigator at the referring institution at Oncotech where the EDR was performed during this time frame was 85%. Their findings also supported the notion that

platinum resistance may be the principle determinant of outcome in patients treated with platinum combined with other agents currently available for first line use. Twenty-two percent of chemotherapy naïve, advanced ovarian cancer patients evaluated in this study demonstrated in vitro platinum resistance, a rate similar to percentages of clinical resistance to platinum or platinum combinations in clinical trials of advanced ovarian cancer (Colombo, 2000). The finding that in vitro platinum resistance was a significant determinant of survival for ovarian cancer patients parallels a vast amount of clinical data suggesting that patient response to platinum is the principal determinant of outcome. The adjusted relative risk for death in this study for platinum-resistant patients was 3.71-fold higher than for nonresistant patients with a relative risk for progression that was 2.6-fold higher.

EDR assay results were available prior to chemotherapy selection. Patients were treated with platinum regardless of their in vitro platinum response. Paclitaxel was the second agent administered unless the patient's tumor showed greater in vitro resistance to paclitaxel than to 4HC or doxorubicin. If so, substitutes were administered. Patients who demonstrated in vitro resistance to paclitaxel and/or non-cross-resistance to doxorubicin received cyclophosamide in combination with platinum (CP); cyclophosamide, doxorubicin, and platinum (CAP); or platinum alone. Median progression-free survival (PFS) was 6 months for the 17 cases exhibiting EDR to platinum, compared to 24 months for the 62 cases exhibiting LDR to platinum in vitro. Overall five-year survival was 19 percent for patients treated with platinum or cisplatin alone. In vitro platinum response remained an independent predictor of PFS and outcome survival in multi-variate analysis

adjusted for cyclophosamide plus platinum versus paclitaxel, CAP, or platinum administration.

The rationale for determining patient classification as platinum resistant using the EDR assay was based in part on an evaluation of the Oncotech database of more than 11,000 cases where these two agents were tested in parallel on the same specimens. Meta-analysis and several randomized trials have demonstrated equivalent efficacy or the two platinum compounds in the treatment of ovarian cancer (Aabo et al., 1998).

Other researchers have independently confirmed the accuracy of the EDR assay for ovarian cancer. Andreotti et al. (1995) correlated clinical response of 70 untreated and 30 refractory ovarian cancer patients with in vitro response using luminescence assay. More recently Taylor et al. (1998) studied 37 ovarian cancer cases using the MTT assay, and found that 65% of patients in the low drug resistance group had a complete response, compared to only 15% in =0.005. Konecny et al. (2000) evaluated the ATP assay. Each of these studies support the ability of in vitro assays to predict clinical response to drugs prior to their administration and that the most robust predictive probability appears to be the identification of inactive drugs rather than the identification of drug sensitivity. These studies are consistent with others that have evaluated survival differences between patients receiving in vitro-resistant versus in vitro-sensitive agents, and suggest that agents found to be inactive in vitro are unlikely to produce clinical responses or improved survival (Freuhauf, 2002).

In one study by Fruehauf and Manetta (1994), the investigators wanted to more fully evaluate the importance of the MDR-1 expression in human ovarian cancer, and its potential relationship to taxol resistance, using the EDR in vitro test. Resistance to taxol

appeared to be significantly increased in MDR-1 expressing ovarian tumors. Those showing LDR in the assay had clinical response rates (≥50% reduction of tumor size as measured in two dimensions) which demonstrated an approximate one and a half to two-fold better responses than the overall response rate. Patients with IDR had a response rate of half the overall rate, while the EDR group had less than a 1% clinical response rate. The conclusion of this study, that the lowest degree of ovarian cancer resistance was seen in untreated cases, is consistent with the general view that a significant proportion of drug resistance in ovarian cancer is acquired. Those who had been treated with first-line drugs and the second-line agent were found to express a significant degree of drug resistance to both. Comparing untreated with treated patients; it was of particular interest that both second-line taxol and first-line cisplatin showed a 7 to 10% increase in EDR.

Twenty-two of the ovarian tumors tested were MDR-1 positive, and the expression of MDR-1 increased from 19 percent for untreated patients to 29% for patients who had previously received chemotherapy. They found that MDR-1 tumors were approximately twice as resistant to taxol. The study supported the notion that expression plays a significant role in both intrinsic and acquired taxol resistance. It was noteworthy that PSC833 was capable of complete reversal of taxol resistance (Holloway et al., 2002).

One study (Orr et al., 1999) examined the feasibility of using the in vitro assay for drug resistance to guide treatment after cytoreductive surgery with a 24 month follow-up. The study involved 66 patients with advanced ovarian cancer. Patient inclusion criteria included histologic confirmation of epithelial ovarian stage III cancer, no prior

chemotherapy or radiation therapy, no co-existing neoplasm, and optimal residual disease (<2cm). Malignant tissue from the involved ovary of each patient was tested in vitro for drug resistance, and chemotherapy was directed individually by assay results. On the basis of the assay 19 patients were treated with platinum/paclitaxel (TP) and 47 with platinum/cyclophosphamide (CP). Three-year survival was 69%; the 95% confidence interval was 58 to 80%. There was no difference in three-year survival between the 19 patients treated with TP (66%) and the 47 patients treated with CP (74%).

The cost-effectiveness of these treatment options was also examined. It cost $4615 to achieve 3-year survival for patients receiving CP and $17, 988 to obtain a similar survival with TP. The cost-effectiveness of assay-directed therapy was $9768 (Orr et al., 1999).

In Vitro Assay Testing for Brain Metastasis

Although the literature contains many reports of the application of drug resistance assays, little is known about extreme drug resistance in primary brain tumors. This is yet another example of a malignancy that certainly appears to exhibit extreme drug resistance. Through the continued analysis of brain tumor specimens and compilation of data from multiple institutions, chemoresistance profiles can be compiled to assist in the development of rational clinical trials and treatment regimens for patients.

One study, Haroun et al. (2002) took this approach. From September 1991 to February 1998, 64 brain tumor specimens were collected from patients admitted to Johns Hopkins Hospital. Tumors were classified according to the revised World Health Organization system. Brain tumor specimens were tested against 13 different chemotherapeutic agents using an EDR assay. Extreme drug resistance was displayed in

many of the tumor samples to the most commonly administered chemotherapeutic agents. This study demonstrates the potential for EDR in vitro assays as an aid in more educated selection of chemotherapeutic regimens.

Brain metastases are clinically diagnosed in up to half of the patients with metastatic melanoma and in 15-20% of the patients the brain is the first site of relapse; this is a major cause of morbidity and mortality (Douglas and Margolin, 2002). The prognosis of patients with melanoma brain metastasis is poor with a median survival time of six months after diagnosis. One study which sheds light on the intricate challenge of the biology of the brain once melanoma metastasizes was conducted by Fidler et al. (1999). These investigators injected different murine or human melanoma cells into synergistic or nude mice to produce melanoma in different regions of the brain. This site-specific metastasis is not due to patterns of initial cell arrest, motility, or invasiveness, but rather to the ability of melanoma cells to proliferate in the brain parenchyma or the meninges. The blood-brain barrier is intact in metastases that are smaller than 0.25 mm in diameter. Although in larger metastases the blood-brain barrier is leaky, the lesions are resistant to many chemotherapeutic drugs. This study also analyzed the malignant behavior of several melanoma cell lines isolated from brain or visceral metastases of patients. The cells from brain metastases showed a slower growth rate and exhibited lower metastatic potential than cells from visceral metastases, indicating that brain metastases do not necessarily represent the end stage in the metastatic cascade. Rather, brain metastases are likely to originate from a unique subpopulation of cells within the primary neoplasm.

In an adjunct to a prospective phase II blinded study of 48 patients with recurrent malignant glioma, Parker et al. (2004) evaluated the predictive reliability of EDR to identify clinical resistance to irinotecan (CPT-11) using fresh tumor biopsies obtained from recurrent patients immediately prior to their first dose of CPT-11 therapy. A 13 week median survival for EDR cases was significantly shorter compared to 38 weeks for IDR/LDR cases. In the 100-day follow-up of these patients, survival favored the IDR/LDR cases. The investigators concluded that these prospective data support the notion that patients should avoid agents to which their tumor demonstrates EDR (Parker et al., 2004).

Chemoresistance Testing In Malignant Melanoma

There have been very few studies that evaluate the effectiveness and accuracy of EDR testing for malignant melanoma. The purpose of the research study contained in this document is to offer data that may underscore the need to consider further evaluation of the role of EDR testing in malignant melanoma. Schandendorf et al. (1994) conducted a retrospective comparison between in vitro drug testing results and clinical response. The investigators concluded that in vitro drug testing may increase the likelihood of obtaining clinical response in the treatment of disseminated malignant melanoma. The major limitation in the treatment writes the lead investigator Schandendorf, was "the lack of availability of effective agents for treatment" (Schandendorf et al., 1994).

Prignano et al. (2002) evaluated metastatic melanoma cells (MMCs) from five patients. Immunoflurosence and electron microscope studies were performed in order to establish the ultrastructural and physiopathological features of MMCs. No significant growth inhibitory effects (≤25%) were observed with INF alpha-2a concentrations up to

8000 IU/ml. MMC's expressed progression markers typical of cutaneous metastatic melanoma and showed poor sensitivity in vitro to most anticancer drugs tested, including temozolomide.

Myatt et al. (1997) utilized in vitro testing in their research with uveal melanoma which has a high mortality rate and responds poorly to all existing chemotherapy. The testing was performed on some agents who had not been used against this tumor. In vitro testing was used to measure the effect of nine cytotoxic drugs at multiple dilutions in 28 primary uveal melanoma specimens. Evaluable assay results showed variable sensitivity to alkylating agents (3 of 9 with mitomycin C, one of 15 with cisplatin and 7 of 15 with treosulfan), sytosine arabinoside (7 of 16), paclitaxel 1of five) and doxorubicin (2 of 16). No tumors were sensitive to temozolomide or 5-flurouracil, and only one of 14 to vincristine. The combination of treosulfan with sytosine arabinoside resulted in enhanced tumor cell inhibition in three of five tumors tested. Combinations containing paclitaxel combined with doxorubicin or cisplatin showed some activity, but none achieved 100% inhibition and the results were similar to those obtained with paclitaxel alone. Clinical trials are underway looking to replicate these findings. Thus, the use of in vitro testing provides a method of testing multiple agents and combinations in a way which would be otherwise impossible in rare tumors such as uveal melanoma (Myatt et al., 1997).

In a study conducted by Cree et al. (1999), the considerable heterogeneity of the chemosensitivity of metastatic cutaneous melanoma was demonstrated. Melanoma specimens from 55 skin or lymph node samples were tested by the in vitro method at three different laboratories. Several of the common chemotherapeutic drugs used to treat melanoma were used. The degree of heterogeneity observed suggests that the in vitro

drug resistance testing can be used to select patients who might benefit from specific chemotherapeutic agents alone or in combination (Cree et al., 1999).

There are promising studies in consideration of the advantages in vitro testing may offer for biomarker detection in malignant melanoma. The role of in vitro testing is likely to continue to shed light on the challenges confronting both scientists and the treating physicians. Some of the advantages and disadvantages at present with the assays follow.

Clinical Benefits Derived From Utilization of In Vitro Assays

There are a number of factors influencing the clinical utility of in vitro assays. Chu and DeVita (2001) provide an extensive discussion of the general issues involved. Relevant to the discussion here, clinical correlations of in vitro tests can be evaluated with the standard statistical tests for sensitivity, specificity, and positive and negative predictive probability. Ultimately, the accuracy of these methods is assessed with three main criteria (von Hoff, 1987):

1. Retrospective comparison of test results with patient response to therapy clinical correlations.
2. Retrospective comparison of test results with patient survival.
3. Randomized prospective controlled clinical trials comparing standard therapy with chemotherapy chosen by in vitro assay results.

The first two comparisons are the normal methods by which laboratory tests are calculated or assessed for accuracy. Many have called for prospective clinical trials for EDR testing even though other laboratory tests (the results of which also guide clinical decision making) seldom are assessed this way (Weisenthal, 1993; Browman and Preisler, 1992; Sampson et al., 2004). According to Kern and Weisenthal (1992),

randomized trials are not required to prove that physicians should not administer inactive drugs and that existing cell culture assays are acceptably accurate in identifying inactive drugs (Weisenthal and Kern, 1992). Weisenthal and Kern (1992) advocate the utilization of cell culture assays to choose between different forms of therapy that would otherwise be equally acceptable in the absence of test information.

A review of clinical correlations for a number of assays identified more than 4200 comparisons of in vitro assay results with patient outcomes (Bosanquet, 1994). Per Freuhauf and Bosanquet, pg. 11 (1993), "it is clear that the reliability of predicting drug resistance with in vitro tests, regardless of the method, is generally greater than 90%." The reliability of predicting sensitivity in the Bosanquet (1994) review was as high as 72%. The reliability of all tests is determined by the prevalence of what is being evaluated in the population.

Given that 70% of solid tumors are resistant to chemotherapy (Freuhauf and Bosanqeut (1994), it is natural that identification of resistance is more accurate than identification of drug sensitivity, whereas the converse can be true for leukemia. From the standpoint of clinical utility, reliability is a prerequisite to make sound management decisions. The ability of in vitro drug response assays to detect drug resistance with an accuracy of 99% compares favorable with antibiotic sensitivity testing. Reported correlations between antibiotic resistance in vitro and patient response range from 67 to 96% (Gawan, 1974; Sanders and Sanders, 1982; Jones, 1982; NIH consensus development conference, 1980; Freuhauf and Bosanquet, 1993). Predictive values for antibiotics are considered acceptable if correlated with patient response more than 80% of the time. Drug sensitivity determinations for antibiotic testing are generally more

accurate than those for in vitro cancer drug response because of the superiority of the antibiotics compared with antineoplastics.

A fundamentally important criterion for evaluating in vitro drug-response tests is their correlation with patient survival. Retrospective and prospective trials have been performed to this end. In some cases, patient therapy was guided by the assay results to determine whether a superior outcome would be gained from this approach (Gazdar et al., 1990; Von Hoff et al., 1990; Yamaue et al., 1996; Wilber et al., 1992; Freuhauf and Bosaquet, 1993). Of the 17 studies evaluating survival, 12 showed a statistically significant advantage for assay-sensitive patients. Three prospective studies, two of which were randomized, showed a survival advantage for patients treated with assay-directed therapy (Gazdar et al., 1990; Yamaue et al., 1996; Wilber et al., 1992; Freuhauf and Bosaquet, 1993). These encouraging reports promoted the larger studies evaluating these in vitro drug response technologies with a variety of cancer types.

The clinical utility of any test must also be judged by how it influences patient care. For a test to be useful, its results should modify patient care in a significant proportion of cases. Because 70% of solid tumors are resistant to chemotherapy, the applicability of drug resistance testing is quite high; however, these tests must be suited to the clinical setting, where a great deal has been learned from epidemiological studies about which agents are most active for specific histologic types. Based on the fact that drug resistance is usually not an all-or-nothing phenomenon, in most cases standard drug regimens still can be administered to patients who are resistant to one of the standard drugs. For example, a patient with breast cancer found to be resistant in vitro to doxorubicin has a greater than 80% probability of being responsive in vitro to 5-

flurouracil or cyclophosphamide (Robert, et al., 1992; Fruehauf and Bosanquet, 1993). For patients with ovarian cancer who are found to be resistant in vitro to cyclophosphamide there in high likelihood that they will be responsive to cisplatin or carboplatin (Eck, Pavich and Freuhauf, 1993; Fruehauf and Bosanquet, 1993). In both of these cases, a standard therapy regimen can be designed that excludes the inactive drug, and it can be given with a reasonable expectation of patient response. Avoidance of inactive agents decreases the probability of patient morbidity secondary to a drug that provides no benefit (Weisenthal et al., 2003).

The application of chemosensitivity assays to diseases for which standard treatments are available (e.g. breast carcinoma) may point oncologists toward ad hoc, nonstandard treatments if the two or three "best" drugs are picked for a particular patient. In addition to the poor accuracy of chemosensitivity assays, ethical and medical-legal issues make the oncologist or clinician wary of this approach. The EDR assay, however, can be used with standard guidelines of oncology practice to eliminate ineffective single agents when the probability of clinical response is highly remote (less than one percent).

The benefit to clinical validation in the EDR assay is avoidance of unnecessary treatments and toxicity. Brown and Markman (1996) conclude that "when one drug is eliminated on the basis of chemoresistance assays, it is implied that the remaining drugs, which do not show chemoresistance, are potentially more effective" (Brown and Markman, 1996). This may be overstating and overlooking potential benefit of in vitro drug resistance testing. For a particular patient, the goal of the EDR assay is in identification of drugs that will not be clinically effective. Other therapies may also be ineffective in that patient, even if those drugs do not show extreme drug resistance in

vitro. Still as ideal as EDR assays appear in present studies with malignant melanoma, it may be that identification of a drug that kills every cancer cell in the Petri dish may be ineffective in the patient. More data is needed to continue to explore accuracy of EDR.

Some oncologists argue that improved patient survival is the relevant outcome for chemoresistance assays. Others may state the converse is true. Patient preferences may dictate that quality of life for the duration of the disease may favor therapeutic attempts to increase survival. This issue is considered paramount to the physicians who treated patients in the Yale Cancer Center Melanoma Unit as discussed further in the document in sections describing the evaluative study.

Clinical failure is the relevant outcome for chemoresistance assays. Clinical response is necessary, but not sufficient, to achieve prolonged survival in cancer patients. Without a response, therapy is ineffective. Proponents of the EDR assays believe the ability to predict clinical failure with 99% accuracy is the distinguishing feature of the EDR assay (Wieland, 2005; Nagourney, 2005) yet others (Schrag, 2005) find no compelling evidence that chemosensitivy or chemoresistant assays should be integrated into routine oncology care until further randomized testing is conducted. If one wanted to conduct a randomized trial of the EDR assay with survival as the outcome, the assay-directed arm would have patients treated with only drugs to which their tumors had the EDR. A statistically shortened overall survival would then be the relevant endpoint. There remains controversy and further testing is needed to adequately endorse complete inclusion of the EDR assays in the physician treatment plan for malignant melanoma. However to date there is enough data to warrant continued pursuit into its contribution

especially as progress is realized with in vitro testing with biomarkers and tumor resistance identification.

Brown and Markman (1996) considered the question of whether the elimination of ineffective therapies is sufficient to justify the routine use of drug resistance assays. The answer depends on the particular clinical situation as it does for every other test and intervention in medical practice today.

The EDR assay finds its greatest utility in settings where (1) the oncologist is considering several drugs or treatment regimens for a particular patient; (2) the treatment options being considered have significantly different toxicities and costs; (3) no standard regimens exist; and (4) refractory cancers are being treated, for which options include salvage therapy, experimental therapy, referral to a clinical trial, or supportive care. Smith and Bodurtha (1995) argue that the avoidance of unnecessary suffering, injury, or harm should be considered in oncology decision-making, because "Beneficence or doing good is enmeshed with nonmaleficence, avoiding harm". In addition, Smith (1996) points out that, "The present crunch in health care economics will dictate that we cannot spend money on ineffective therapies." The EDR assay accurately identifies ineffective chemotherapy and helps the oncologist avoid unnecessary treatments and toxicity.

Disadvantages of In Vitro Assays

The issues limiting the widespread use of in vitro chemosensitivity testing are: (1) in vitro drug testing is relatively expensive and time consuming; (2) the efficient procurement of tumor tissue and technical issues related to its successful growth in vitro remain serious problems, with only about one-half or tumor samples from 12 clinical trials having enough cell numbers for drug testing; and (3) only one-third of all patients

entered in prospective trials of in vitro drug testing are actually treated with the in vitro best regimen. (4) Inability to mimic the in vivo environment is another concern regarding utilization of the assays. The next sections will address these limitations.

There are many technical limitations to in vitro assays of drug response. First and foremost among them is whether the whole tumor is represented by the tested cells (Shaw et al., 1996). This concern is related to the importance of stroma (fibroblasts, endothelial cells, etc.) in tumor survival and drug susceptibility. Some have suggested that tumor stroma is part of the tumor (i.e. transformed), genetically different from normal stroma and should appropriately be reflected in standard test effectiveness measures such as PCI. Others argue that the presence of these cells reduces the accuracy of the tests as they can take up the substances used to measure the experimental endpoints of the assays.

Standardization of the techniques from one setting to another is also important. In order to have comparable results across specimens, handling must be consistent in the clinical and laboratory settings. One issue related to standardization is how the tumor tissue is processed after surgical removal (Gercel-Taylor et al., 2001). Obviously, the tissue is exposed to a multitude of factors-in transit from the body to the Petri dish-that may ultimately influence in vitro performance. Concerns about the ability to replicate the entire assay process from one sample to another have received mention in the literature.

Within a scientific setting, the duplication of assay results is often difficult if an experiment is not precisely replicated. Lieberman et al. (2001) demonstrate this point in the context of EDR assays. They (Lieberman et al. (2001)) conducted a series of experiments that demonstrated that the concentration of cells used in the assays is a critical parameter for determining the activity of a particular toxin. Thus, one cannot

assume directly proportional inactivity between assays of the same toxin assessed at different cell concentrations. The Lieberman group also provided evidence that the concentration of the drug-toxin is an important parameter, stating that comparisons of cytotoxicity data obtained from different laboratories may be problematic unless the precise conditions under which the assays were conducted are stated which is not always the case in the literature. In addition, there is a high degree of variability regarding the exact amount of time cells are in culture from one study to the next.

Inability of in vitro assays mimicking the in vivo environment

Another obvious drawback of in vitro tests is that none of these assays are truly capable of mimicking the in vivo environment. Very little information is available regarding intracellular and interstitial drug levels in vivo, not to mention the importance of tumor vascularity. Drug delivery to poorly vascularized regions of tumors is an inadequately understood topic, particularly because there is high variability in the vascular make-up of tumors. The blood supply to many solid tumors becomes insufficient and intermittent resulting in regions of hypoxia, low extra-cellular pH, and reduced cell proliferation rates. This ultimately affects drug efficacy. Cytotoxic drugs must diffuse from the blood vessel, and then through multiple cellular layers, in order to reach their target cells (Phillip et al., 1998).

The effects that chemotherapeutic and immunologic therapies exert upon the human body are many as discussed in the section about chemotherapeutics and immunotherapy. The drug effects are potential complications to existing underlying disease states or can alone be debilitating of even fatal. These effects are seen in vivo but not in vitro. At present these systemic effects that can elicit many problems for the patient

and the treating physician in managing and monitoring sustained progress are not adequately reproducible in the laboratory setting. As the treating physician must consider potential side effects when administering therapeutic agents, the in vitro test does not factor in as augmentation to this aspect of management consideration.

Moreover, each patient has a unique pharmacogenetic makeup. There are significant inter-patient variations in drug half-life, volume of distribution, types of metabolites formed, protein binding, and routes of elimination (e.g. renal excretion hepatic metabolism). Also, there are many chemotherapeutics that require metabolic activation via liver metabolism (P450 system). There are possible effects that occur from the agents administered for the treatment of advanced melanoma that are seen in vivo but not in vitro.

For many years, all efforts to treat cancer concentrated on the inhibition of growth or the destruction of tumor cells. A strategy of both eradication of tumor cells by chemotherapy and immunotherapy and modulation of the host environment (e.g. tumor vasculature and hypoxia) is an additional, relatively novel approach to cancer treatment. Drugs acting on tumor cells which have a metastasis-prone mutational or expression status (by classical or targeted chemotherapy) as well as drugs affecting host-mediated survival pathways must be combined in order to create therapeutic synergy. Therapeutic maneuvers may target receptor tyrosine kinases, chemokines or protein-coupled receptors in tumor cells. Moreover, stromal and immunological cells and their cytokines coordinate critical pathways that exert important roles in the ability of tumors to invade and metastasize, thus suppressive cytokines and neutralizing specific antibodies might subvert conditions for metastasis. These conditions may be found in vivo but may not be

observed or measurable in vitro. The next sections discuss in vitro testing, clinical survival and cost effectiveness.

Outcomes of in vitro testing and their effect on clinical survival

When clinical studies of in vitro drug sensitivity tests are viewed as a whole, the potential clinical benefit is not entirely clear. Based on the available data, it appears that in vitro-selected chemotherapy regimens may be as good as empiric regimens, but not necessarily superior (Cortazar and Johnson, 1999). The study in this document addresses this very issue as exemplified in the study outcome Chapter 3).

In five trials of patients treated with the in vitro-best regimen (IVBR) versus physician-based empiric chemotherapy, two showed that survival was 4 and 19 months longer in the IVBR arm (Cortazar et al., 1997 and Von Hoff et al., 1990). One showed that survival was 4 months shorter in the IVBR arm (Shaw et al. 1996). Two of the trials demonstrated equal survival times for IVBR and empiric treatment (Gazdar et al., 1990 and Von Hoff et al., 1991). However, it has been suggested that the clinical validation of in vitro chemoresponse assays must not necessarily be based on survival alone but also on how well the in vitro sensitivity/resistance assay correlates with the in vivo experience (e.g. tumor regression, and in the case of chemoresistance assays, avoidance of unnecessary toxicity) (Brown and Markman, 1996).

Cost of In Vitro Testing

Dickenson and Myers (1990) evaluated the estimated cost savings from avoiding ineffective therapy in Rhode Island. They found that the cost for an average patient with cancer was two-fold higher when ineffective therapy was given. When they considered the increased cost burden from patients who survived longer, they found that the cost of

ineffective treatment remained greater than the cost of effective treatment through 10 years of breast and ovarian cancer. Clearly, the medical community is facing a period of increasing cost controls. Additional studies to evaluate the degree of improved resource utilization through the use of in vitro drug-response testing are needed.

Bosanquet et al. (1999) argued in their study that while the cytotoxic antimetabolite fludarabine is a widely used active agent in chronic lymphocytic leukemia (CLL), "cost and occasional adverse side-effects necessitate careful use. Identifying before treatment patients not likely to benefit from fludarabine could advance disease management both clinically and financially." It is an expensive treatment. The article goes on to state that in 1999 the cost per course was approximately $11,000. This drug has been shown to induce more responses and longer disease-free survival (Sorenson et al., 1997; Keating et al., 1991); however, the French Cooperative Group on CLL (1996) recognized the possibility of the increased quality of life with fludarabine but argued its effect on overall survival had not been shown. The traditional first-line therapy is chlorambucil with a lower cost of $700 per course. Traditionally, treatment has been based on clinical trial results and targeted to a cohort of patients with individual variation being catered to empirically.

Mason et al. (1999) made an economic assessment of the DiSC assay in CLL. It concluded that the DiSC assay may be a cost-effective technology for improving patient outcome: the estimated incremental cost effectiveness was $2400 per year of life gained (Mason et al., 1999). Of note, this work was one of the largest series of ex vivo response assays as of 1999 that investigated a single agent in one malignancy paralleling many other such studies concluding that assay guided therapy could enhance and guide

physicians in disease management and treatment options and possibly improve survival rates for this intractable leukemia (Bosanquet et al., 1999).

Smith and Bodurtha (1995) have argued that avoidance of unnecessary suffering, injury, or harm should be considered in oncology decision making. In addition, Smith has pointed out that the current economic environment in health care in the United States will dictate that money cannot be spent on therapies that are not effective (Smith and Bodurtha, 1995; Smith, 1996).

The cost of treating metastatic melanoma per month is approximately $7000/month according to data collected from the evaluative study. As the number of elderly rise in the U.S., the need to address the increase number of cases of malignant melanoma that are likely to also rise as a result of people living longer. Studies directly relating to efficacy of assay directed therapies and treatment effectiveness and cost savings may follow that rise.

Changes in Medicare reimbursement have reflected the consideration of the utilization of in vitro drug testing as evidenced by reimbursement for Oncotech EDR testing serves within the state of California (Medicare, 2000). These changes may result in like changes in other states. Similarly, this may also create pressure for private insurers to pay for chemoresistance testing as well. Payments by insurers whether they are publicly or privately funded may expand the utilization of in vitro drug resistance testing by physicians. To the extent they prove effective in reducing the use of ineffective therapies, they may prove a benefit in the reduction in direct and indirect cost of disease management in malignant melanoma.

Conclusion to Chapter 2

Studies have demonstrated that in vitro tissue micro array testing is a diagnostic test that can help identify the uniqueness of a given patient's tumor and therefore the drugs which may yield resistance. Data from several studies have indicated that chemoresistance assays may be used to select patients who might benefit from an individually adapted cytostatic therapy (Ugurel et al., 2003). Clinicians who treat malignant melanoma have the option to utilize these diagnostic tests. There are many studies presently being conducted which evaluate the feasibility and predictive value of chemosensitive and chemoresistance in vitro assay testing in melanoma patients. As stated by Jaeschke et al. (1994) "the ultimate criterion for the usefulness of a diagnostic test is whether it adds information beyond that otherwise available, and whether this information leads to a change in management that it ultimately beneficial to the patient" (Guyatt et al., 1986b; Jaeschke et al., 1994).

Schattner et al. (2004) found that patients want their physicians to be highly professional and expert clinicians and show humaneness and support, but their first priority is for the physician to respect their autonomy. Previous research has already identified the complexity of patient's needs in the modern era. A relatively recent systematic review of the literature on patients priorities, found "humaneness" to be the most highly rated aspect of care, followed by clinical competence and patient participation in decisions. Patient's preferences remain integral to modern evidence based practice (Haynes, Devereaux and Guyatt, 2002; Schattner et al., 2004). Patient needs are variable and include quality of life issues, cost of healthcare, and underlying health status. When consideration of treatment is given for a disease that is life-threatening, the patient relies on good judgment from their clinician. Good judgment

depends on maximizing the delivery of good quality health care to the patient and minimizing clinical error.

Errors can never be eradicated, unfortunately, because new diseases emerge, tests are never perfect, patients are sometimes noncompliant, and clinicians will inevitably, at times, choose the most likely diagnosis over the correct one, illustrating the concept of necessary fallibility and the probabilistic nature of choosing a diagnosis. System errors play a role when diagnosis is missed or delayed or treatment choice is incorrect due to latent imperfections in the health care system. These errors can be reduced by system improvements, but can never be eliminated because these improvements lag behind and degrade over time, and each new fix creates the opportunity for novel errors. Tradeoffs also guarantee system errors will persist, when resources are just shifted. Cognitive errors reflect misdiagnosis or treatment from faulty data collection or interpretation, flawed reasoning, or incomplete knowledge. The limitations of human processing and the inherent basis in using heuristics guarantee that these errors will persist. Conscience compels one not only to oppose and disobey an unjust law but also to seek to hinder its enforcement (Baumann, 1993). Opportunities exist, however, for improving the cognitive aspect of diagnosis and treatment. Inherent in the practice of medicine is the goal of improving quality of life and when feasible, the extension of life and avoiding harm. As excerpted from the Hippocratic Oath (Edestein, 1943):

"I swear by Apollo Physician Asdepins & Hygieia and Pacaceia & all the gods and goddesses, making them my witness that I will fulfill according to my ability and judgment this oath and this covenant: I will apply dietetic measure for the benefit of the sick according to my ability and judgment. I will keep them from harm and injustice. I

will neither give a deadly drug to anybody who asks for it, not will I make a suggestion to this effect. In purity and holiness I will guard my life against it. If I fulfill this oath and art and do not violate it, may it be granted to me to enjoy life and art."

Keeping in mind the insidious nature and rapid spread of malignant melanoma, one can realize that when facing therapeutic decision making on a case by case basis, reality favors the ideal. Chapter 3 presents the clinical practice particular to the physicians in the Yale Cancer Center Melanoma Unit and consideration and utilization of in vitro testing in the therapeutic process for malignant melanoma.

Chapter 3 discusses the evaluation made for the utilization of in vitro drug assays conducted at Oncotech Inc Laboratory for a small cohort of patients who were treated by surgeons and physicians in the Yale Cancer Center Melanoma Group for the period of time between 1994 and 2004. This study evaluated decision making and associated variables involved in the clinical utilization of the testing methods in order to form conclusions regarding treatment determination.

CHAPTER 3: RESEARCH EVALUATION COHORT AND OBSERVATIONS OF THE YALE CANCER CENTER MELANOMA UNIT; UTILIZATION OF EDR IN VITRO ASSAY AND MALIGNANT MELANOMA

The preceding chapters have established the rapid progression of malignant melanoma as well as the absence of effective therapeutic interventions. While existing therapies have not led to cures or durable remissions, it is nonetheless desirable to avoid the use of drugs to which the disease of specific patients exhibits drug resistance and which is likely to be related to shortened survival. However, the rapidity with which malignant melanoma progresses to death makes immediate treatment common. This style of treating the disease first and waiting for confirmatory test results can in theory result in reduced patient survival if drugs chosen are those to which an individual patient's tumor is resistant. Several considerations in addition to survival potential comprise therapeutic consideration for the treating physician regarding advanced melanoma. Quality of life and economic burden are issues relevant to such consideration. Chapter 4 will discuss features of clinical consideration regarding heuristic practice as well as incorporation of evidence-based medicine. There are a few studies cited from the literature which have examined patient preferences with regard to confidence of physician practice methods as it affects patient compliance to the medical regiment. This information and such studies are relevant in a disease that claims life quickly.

Approach to Treatment Plan for Malignant Melanoma: Current Studies in Medical Literature

There are no studies in the literature which support patient preference regarding therapeutic choice for metastatic melanoma. The study, from the patient perspective

would be most representational if conducted retrospectively. This is not possible given it is not feasible to learn once the disease has taken a patient's life. On the front end if a physician can obtain additive information as to whether an individual patient has a tumor which is resistant to existing chemotherapy or immunotherapy, it may indeed provide more information for the overall treatment recommendation. Quality of life is a subtle and subjective parameter and clearly varies with each patient. It is not only difficult to measure experimentally to then incorporate findings into evidence based medicine it is next to impossible to know in advance whether the agents currently available for advanced melanoma will in fact substantially alter quality of life. To one patient quality of life may be altered by days off from work secondary to the extreme nausea often resulting from interferon or may severely worsen an underlying systemic disease if blood dyscrasias result from IL-2 administration necessitating further stay in the hospital. The displacement of one's living situation may be altered due to the need to participate in a clinical trial in a state other than where the patient resides. All of these common possibilities exist for the patient with advancing melanoma. Each scenario also carries with it a potential economic burden. The real difficulty is that the decisions made both by the treating physician and the patient and patient's family in most cases must be made rapidly to try to stay ahead in some fashion, of the devastation of the tendency of the malignant behavior of the disease. Each patient would likely respond differently if given the option that based on previous cases, their course of illness before demise may indeed be effected by the administration of available therapies. It may be possible that as examination is made as to the accuracy and effectiveness of in vitro drug resistance testing and biomarker identification for malignant melanoma, this data could offer more

information for the decision making process. Presently the tests are not tried enough nor have their efficacy for malignant melanoma been proven. It is possible through further testing they may gain the respect necessary to sustain utilization in more routine clinical use and could thus become invaluable.

End of life issues when placed before a patient unexpectedly demand unreasonable questions, never-the-less the questions are those that the treating physician must contemplate and must help the patient with. The mindful physician considers these questions and through their practice style affords as much data to the patient as is available. This practice style was a constant observed in the study as maintained by multi-disciplinary group at the Yale Cancer Center Melanoma Unit. There is neither a standard nor clear evidence-based path by which to drive the individual therapy plan when considering advanced melanoma. There are any number of variations on social, physical, economic, psychological and ability to understand conceptually all that occurs from the moment a patient receives the diagnosis through to the likely death. Six or eight months are relatively short when many factors are at stake. The treating physician is relied upon to guide the patient. An August 13, 2005 New York Time's front page article which continues for three full pages documented a young woman's struggle with the diagnosis of ovarian cancer. The theme of the article depicts months of personal journey to find the best doctor and the best source to gather answers and guidance as to the ideal course for her individual case. In the end as the woman wishes the public to know, her trust lie with her primary care doctor and the words of advice and guidance from this source. The article depicts the desire of the individual to forge their own path to know which drug to take and when and for how long. The woman in the article revealed her

tireless attempts to self-inform through internet medical articles and through the fleet of various physicians who are specialists. The resounding conclusion to the article as captured by the woman is that the fight against cancer has no definite answers but what brought her the most security (and could be characterized) as quality of life, was the physician who spent time explaining the evidence as it is available, her options of therapy but packaged with an understanding of her particular needs. There are no standard guidelines when end of life issues confront the individual patient with such haste.

Background Discussion for Observational Study

The medical practice style of the physicians at the Yale Cancer Center Melanoma Unit in their collective approach to the individual patient with inclusion of medical, social, psychological, economic and evidence from previous cases is impressive. The heuristic clinical approach to the individual patient when factoring in underlying health status and social circumstance in devising treatment decision and referral to clinical trial is unique at the Yale Cancer Center Melanoma Unit. There was not a hospital or multi-disciplinary group that functions using a similar clinical practice approach for the treatment of malignant melanoma to serve as a control for study comparison. In this study, an experimental design was not conducted. Rather the retrospective cohort evaluation and evaluative observatory study of the Yale Cancer Center Melanoma Unit serves as an approach that may be utilized as an indicator to match the decision making process within another melanoma unit or as use for theoretical background to propose a model for the development and operation of a melanoma unit or other cancers.

The evaluation of in vitro testing of tumor tissue removed from the patient with advanced melanoma to determine extreme drug resistance and or biomarker identification

as additional information for both the patient and treating physician is considered for this study. As with consideration of all novel diagnostic testing evaluation of accuracy and feasibility of utilization, reproducibility and generalizability and improvement of survival are variables to examine.

The research in the study involved a retrospective evaluation of small study cohort of patients with stage III or IV malignant melanoma, each treated by the same group of physicians and using a multi-disciplinary approach when indicated. Each patient in the cohort had their malignancy tested for in vitro EDR at Oncotech, Inc. Laboratory. The utilization of the EDR test results was evaluated through patient medical records from the cohort group and through observation of the multi-disciplinary group comprising the Yale Cancer Center Melanoma Unit and interview of its members. The question as to whether the EDR results contribute to therapeutic decision making is the primary purpose of the study. Additionally the decision making process employed by the group of treating physicians is discussed and the advantages to such an approach is offered as a model for future similar therapeutic decision making.

The treatment choices made by the physicians in the multi-disciplinary group are considered and discussed in this section of the document. The Yale Cancer Center Melanoma Unit forum provided an opportunity to examine and consider variables that enter into the decision making equation when a team approach is taken to devise individual treatment plans for a disease which progresses most often to demise rapidly. During the short course of disease each individual patient exhibits a wide range of needs varying in medical, social and psychological component.

Data about the inclusion of the in vitro EDR test results as functional in devising a clinical treatment plan was observed in both collection from the medical records from the cohort group and the group of patients under treatment as discussed in the Yale Cancer Center Melanoma weekly conference. These results provide what may be initial discovery of potential utilization of the in vitro test EDR as an element of the clinical treatment plan for malignant melanoma. This may be especially useful as the in vitro EDR test has not been clinically validated for use for malignant melanoma. Oncotech Inc. EDR in vitro testing has been moderately evaluated clinically through experimental design for breast, ovarian and prostate cancer. Studies are cited in this document. This study suggests there is value in eliciting data regarding potential utilization and possible generalizability of a diagnostic test within the clinical setting when the test parameters are consistent with laboratory conditions that may or may not be reproducible or useful in vivo. At present s with malignant melanoma, skepticism exists with regard to the use of EDR in vitro testing for breast and ovarian cancer (Schrag et al, 2004; Nagourney, 2005).

The heuristic multi-disciplinary approach

A question that may arise in this context is whether treatment approaches currently undertaken by treating physicians commonly lead to treatment with therapeutics against which the disease is resistant. If so, it might be advantageous to delay treatment pending test results. Moreover, whether current styles of treatment typically lead to the use of therapeutics against which the disease is not resistant will become increasingly important as new drugs become available that have the ability to more effectively arrest progress of the disease, bring about remission, or lead to an effective therapies or vaccines.

The question of whether rules developed about treatment through heuristic practice lead to outcomes consistent with those based on a larger set of observations has received attention in the medical literature. Examples of observations are cited in this document as is germane to the evaluation undertaken here. Physicians value their independence and enjoy the interactive nature of their profession. They often develop intellectual pathways or 'rules of thumb' in their practices based on their own knowledge, clinical experiences, and the patient's presentation. These learned practice styles, or heuristics may in fact be consistent with treatment regimes built upon larger samples of cases drawn across many practices, i.e. evidence based medicine. It has also been argued that heuristic practice styles lead to incorrect treatments and that this warrants the use of evidentiary tests and rules where possible.

The study includes observations and interviews with the members of the multi-disciplinary group through the continuation of their treatment of patients in their medical practice using the same approach and consideration of the EDR in vitro testing. The evaluation of the two groups does not include a control group as there is not a cohort of patients who did not receive any treatment for their metastatic melanoma. The purpose of the evaluation is as a means for continued study and opportunity to consider study design that may include parameters as contained in this study where feasible and of interest to other investigators.

Yale Cancer Center Melanoma Unit

The Yale Cancer Center Melanoma Unit has been actively functioning for twenty-eight years. The group consists of the physicians who both treated the patients in this study and who have been treating patients with melanoma each for over thirty-five years.

The group composition and its purpose will be further discussed further in this chapter. The group representation includes oncology, general and plastic surgery, clinical and dermopathology, radiology molecular biochemistry and pharmacology. In total, the group is comprised of a dozen members each with faculty appointment at Yale Medical School faculty and medical practice privileges at Yale New Haven Hospital as medical attending staff. Concomitant to clinical medical practice, each member of the Yale Cancer Center Melanoma Group is involved in clinical trials or research with advanced melanoma.

Overview of Research Study

Variables evaluated from the cohort of patients in the study were examined retrospectively via medical records, pathology identification and interviews with the treating physicians. The therapeutic decision making process employed by the Yale Cancer Center Melanoma Unit was evaluated via data gathered from the small cohort of patients, all deceased at the time of the study, and the current group of patients that were receiving treatment at the time of data collection for the evaluative study. All patients in both the small cohort and the current group diagnosed initially or soon thereafter with stage III or IV malignant melanoma. Systematically each was given individual consideration for treatment determination which was achieved in accordance to a clinical heuristic practice approach using both evidentiary medicine and multi-disciplinary expert contribution.

An evaluation of the utilization of in vitro EDR testing by the treating physicians who ordered the tests was made in consideration of the therapeutic decisions making for both the small cohort of patients and the current group of patients who were in treatment at the time the observations of the Yale Cancer Center Melanoma Unit were made. For

all of the patients in both groups, treatment began prior to the availability of the EDR test results. Thus, it is possible to compare ex post tests against the clinical choices made by this particular practice. The members of the group considered utilization of in vitro EDR testing for each patient. The practice style of the group as a whole is one of blended heuristic style balanced against sole adherence to evidentiary medicine. While examination of one practice in this regard will not settle the debate regarding the relative roles of heuristic and evidence based medicine, it offers a model of therapeutic decision making wherein determination of therapeutics is tremendously difficult, drug resistance extreme, drug choice limited and the course of the disease rendered swift.

Time and course of disease are variables that make inclusion of a control group unrealistic. It is hoped that in the future, survival time may be increased as therapy efficacy also increases. It would be difficult to gather a group of patients with stage III or IV melanoma who are willing to receive no treatment from diagnosis to death to serve as control. Alternatively using the small cohort in this study, taking into account in vitro EDR testing and therapeutic decision and course of disease through to death as a model of the result of the multi-disciplinary team approach was used as a pseudo-control from which to study its reproducibility, advantages and disadvantages in the current group of patients using the same model.

The conduct of the study was organized around three hypotheses regarding the utilization and usefulness of EDR tests by the practice. The chapter commences with the hypotheses generated at the outset of the study and a discussion about the hypothesis. The process of the data collection which determines the hypotheses is described as is the evidence regarding each of the hypotheses. Discussion of the observations and interviews

conducted with the Yale Cancer Center Melanoma Unit follows and conclusions are drawn regarding the efficacy of the clinical heuristic employed by the multi-disciplinary group relative to how that practice might have differed had it been driven by an EDR-directed treatment. Overall as is described in the conclusion to the chapter, the clinical decisions of the Yale Cancer Center Melanoma Unit were consistent with tests obtained from Oncotech Inc. Thus, the clinical heuristic evolved in a manner such that treatments were provided which were overwhelmingly consistent with the best available clinical evidence. The results of the study hypotheses follow a recap of the study hypotheses.

Hypotheses:

(1) Clinicians do not use the results of in vitro testing provided by research laboratories.

(2) If in vitro EDR results are used by the clinician, their use will reduce cost and toxicity to the patient.

(3) If in vitro tissue results are used by the clinician, the patient will benefit through increased progression to survival.

Although the general focus of the evaluative study and group observations and interviews conducted was in determining whether the clinical practice of the Cancer Center Melanoma Group resulted in prescribed treatments similar to those that would have been indicated by an assay directed therapy based upon EDR test results from Oncotech Inc., there were other specific operational hypotheses which the study followed in seeking to address this issue.

First, if the results of tests are to effect treatment decision making, evidence is needed to demonstrate utilization. The hypothesis that clinicians do not use the results of in vitro testing provided by research laboratories was not proven in this evaluative study.

Indications in the results suggest that for a small percentage of the patients in the study, the EDR results were utilized by the treating physicians.

Second, if the test results indicated that a tissue sample exhibited EDR to a current treatment regimen, then by changing to an alternative therapy, economic burden may be reduced or eliminated by avoidance of ineffective and costly treatments. Also, if the sample from a particular patient was found to exhibit low or intermediate resistance to a range of drugs, the least costly therapy could be adopted. This hypothesis was not proven in this evaluative study. However it is plausible that the use of EDR tests could result in reduced clinical costs to the individual. Further studies with larger cohorts may indicate cost savings especially using metanalysis studies which may identify subtle economic gains given the short duration from diagnosis of advanced melanoma to demise from the disease. Excluding spurious variables will fortify such study.

The related hypothesis established for this study is that EDR tests would reduce patient toxicity and thus improve quality of life. In cases where tumor tissue from individual patient yields low or no resistance in vitro to several drugs, the treating physician may consider changing treatment drugs if the patient is also exhibiting signs of drug toxicity. As with economic benefit, the avoidance of drugs to which a patient's cancer is found to be EDR, drug toxicity may too be affected or eliminated by the guidance of EDR testing results for the individual patient. While this conclusion may be found in future studies, the hypothesis that if in vitro tissue results are used by the clinician, their use will reduce costs and toxicity to the patient was not confirmed in this evaluative study.

Third, in ovarian and breast cancer, it has been demonstrated that patients who receive drugs when their in vitro assays indicate EDR exhibit shorter survival times. Thus, it was hypothesized in this study that patients who test positive for EDR in the case of malignant melanoma will exhibit the same pattern. The hypothesis that if in vitro tissue results are used by the clinician, the patient will benefit through increased progression to survival was not proven or disproved conclusively. Future evaluations of in vitro testing for advanced melanoma may narrow the gap to prove benefit in the utilization of EDR test results to increase the survival of patients with stage III and IV melanoma.

Purpose of the Study

The specific aim of this study was to determine if current clinical practices as exemplified by the Yale Cancer Center Melanoma Unit would be substantially altered by adopting EDR testing prior to patient treatment and whether patient management may be better served by a multi-disciplinary approach for patients with advanced melanoma. Mortality from melanoma is rising due to its increasing incidence and the lack of availability of effective therapies once it has metastasized. Therapeutic interventions for metastatic melanoma have been disappointingly ineffective. To date systemic therapy includes biologic agents such as interferon and interleukin and chemotherapy. No cytotoxic treatment has been shown to prolong survival. Methods to detect chemoresistance prior to therapeutic intervention can steer treatment towards drugs to which an individual patient's tissue does not show drug resistance in vitro. As more effective therapies become available, discriminating in their usage may become clinically important. Whether the use of a formal test would result in altered treatment practices in

this context yields useful clinical insights as well as information about the relationship between clinical decision making relative to evidentiary tests. A forum that offers several experts in treating melanoma discussing individual patients in group discussion one patient at a time may prove of substantial benefit to the patients being discussed and additionally may offer wider application to other units or new units that may be set up.

Overview of study hypothesis and outcomes

This study evaluates the treatment decisions made within the Yale Cancer Center Melanoma Unit regarding patients with malignant melanoma relative to information contained in EDR tests conducted for those same individuals by Oncotech Inc. Within this comparison, three specific outcomes consistent with hypotheses of the study were assessed: the utilization of test results, cost and toxicity, and survival. Data obtained from the observations and interviews with the members of the Yale Cancer Center Melanoma Unit together with data results from the evaluative cohort provided an opportunity to construct a proposed model which may be useful in clinical settings within which treatment of advanced melanoma occurs. This model is discussed in the concluding chapter.

The evaluative study presented in this thesis document was conducted under the guidelines of the Health Insurance Portability and Accountability Act (HIPPA) in compliance with the law enacted by Congress in 1996 in protection of patient privacy. HIPPA is also known as Kennedy-Kassebaum Bill, named after its creators, Senators Edward Kennedy and Nancy Kassebaum, legislation signed into law by President Clinton. The overall goal of HIPPA is to provide insurance portability, fraud enforcement, and administrative oversimplification of the healthcare industry. HIPPA

was formed out of the growing concerns about keeping healthcare information private, the need to consolidate nonstandard healthcare data, as well as the general consensus to streamline healthcare operations and reduce the cost of providing healthcare services (Beaver and Herold, 2004).

Compliance and its IRB HIC protocol were obtained prior to collection of data to ensure compliance with HIPPA. The collection of data for this study began in February 2002. There were 78 patients in the study with 100 tumor tissue samples. Each of the 78 patients in the cohort selected for inclusion in this study was diagnosed with advanced malignant melanoma prior to 1994. All of the patients were either initially diagnosed with stage III or IV disease or progressed to that level in the time period from 1994 through 2000. All of the patients ultimately progressed to death prior to the conclusion of data compilation.

In addition to the use of the data in this study, the 78 patients in this cohort were also selected for inclusion into a research study at Yale University Medical School wherein all 100 of the tissue samples were tested to correlate Ki-67 expression with the same Oncotech EDR assay results, "Correlation of Ki-67 Immunohistochemistry with Oncotech Extreme Drug Resistance Assay Profiles in Melanoma". The goal of this additional study was to evaluate the use of Ki-67 as a potential biomarker to be used in identifying appropriate recipients of specific, novel therapies for metastatic melanoma. The study also sought to form an efficient paradigm to select potentially useful agents and define appropriate patient populations based on tumor characteristics (Berger, Harigopal, Martens et al., 2003 Appendix A, abstract).

Observations made through weekly intervals at the Yale Cancer Center Melanoma Unit and interviews with its members were conducted during the same time parameter as the data collected from the medical records of the cohort group. To investigate the research hypotheses required the collection of data from patient medical records. This section documents the data collection and tabulation process. This section of the document also includes data from the collected interviews with members of the Yale Cancer Center Melanoma Unit and observation of the multi-disciplinary approach to therapeutic decision making employed for malignant melanoma by the group.

Methodology: Data Collection

The compilation of data was conducted at the offices of Dr. Stephen Ariyan, a board certified surgeon specializing in head and neck cancer and Dr. Leonard Farber, a board certified oncologist specializing in all aspects of oncology clinical practice. Dr.'s Ariyan and Farber are members of the faculty at Yale Medical School, staff members at Yale New Haven Hospital and members of the Yale Cancer Center Melanoma Unit. Dr Ariyan is the director of the Yale Cancer Center Melanoma Unit multi-specialty team at the Yale Cancer Center Melanoma Unit. The patients receiving treatment at Yale New Haven Hospital Oncology Unit received oncology management also by Dr. Harriet Kluger, a board certified oncologist specializing in breast cancer and malignant melanoma, faculty Yale Medical School, staff member of Yale New Haven Hospital and member of the Yale Cancer Center Melanoma Unit. Dr. David Rimm, M.D. PhD. is a board certified pathologist, member of the faculty of Yale Medical School and is also on staff at the Yale New Haven Hospital. Dr. Rimm analyzed the pathology tissue specimens for this study and the study the study of the correlation of Ki-67

immunohistochemistry with Oncotech extreme drug resistance assay profiles in melanoma (Berger, 2004 and Appendix A).

The medical data for each patient studied was maintained in the medical record for the patient located in one or more of the medical offices. All medical charts for the patient from each office were methodically reviewed from a clinical perspective three times. A compilation of data was obtained and written from each chart review. This data compilation was converted to a color-coded computerized layout format for each patient (**Formatted Melanoma Patient Data**) (Appendix D). The Formatted Melanoma Patient Data was translated to tablature format containing all represented cells into the **Full Melanoma Patient Tabulation.** Ultimately the Full Melanoma Patient Tabulations were reduced and converted to the **Concise Tabulated Melanoma EDR Data** (Appendix B). The tabulated data was utilized in two studies: this study for evaluation of the utilization of Oncotech Laboratory Incorporated EDR testing results and a retrospective analysis to evaluate correlation of Ki-67 immunohistochemistry with the Oncotech EDR assay in melanoma (Berger Harigopal, Martens, et al., 2003 Appendix A, abstract).

A procedure was developed to gather data from patient records. Each record was thoroughly reviewed to assess the available data. If specific data was not available within the medical charts, alternate contact was pursued to obtain the data through outside sources. Outside sources included medical offices and/or hospitals where patients received prior medical care. For example, in some cases, contact was made with prior medical practices to verify or to obtain past data as it pertained to the time when the patient had been under a physician's care other than those listed in the study. In other instances data was obtained by accessing larger data bases housed at the Yale New Haven

Hospital, for example via hospital medical records to confirm or obtain laboratory results from prior hospitalizations not contained within the patient record in the private offices. In some instances, no data could be obtained and was reflected in tabulation and adjusted for outcomes (*adjusted for available data). Validation of death and cause of death was obtained through the Connecticut Death Registry under State guidelines. Each patient where data was available was diagnosed with malignant melanoma as cause of death.

All collection of data was conducted at the medical office or hospital where the medical records were housed. The data collection process took approximately 18 months. The compilation of data was obtained and hand written from each patient record review over a period of approximately 12 months. The conversion to a computerized form, the Formatted Melanoma Patient Data, took approximately three months. Further translation to the Full and then the Concise Melanoma Patient Tabulation also took approximately three months.

Data was compiled and written from the medical records. Information collected included patient demographics, medical, family, and social history, serial laboratory written reports, written clinical feedback notes from specialty consultations for melanoma, written clinical feedback notes for specialist consultations for ongoing systemic disease and/or new onset of disease secondary to pharmacologic side effects from chemotherapy, histopathology reports, operating room medical forms detailing surgical procedures, medical notes for additional hospital admissions, imaging study results, death dates, and other data pertinent to this study. When a patient case was presented to the Yale Cancer Center Melanoma Unit Weekly Conference, detailed medical notes were taken by the surgeon, Dr. Ariyan and included in the patient record.

At the initiation of this research, all medical records were reviewed and data from each record was placed into categories to determine both inclusion and exclusion of relevant categories as variables. After the initial chart review for the 78 patients was made, it was determined that additional variables would enhance the validity of the study (addition of LDH serum results, date intervals between initial diagnosis of malignant melanoma, date of recurrence with accompanying side-effects and dose reduction adjustments). The addition of these variables for evaluation prompted a second review of records of the 78 patients.

After these two passes through the charts, a computerized form was created to insert the data into the computer using a color-coded Formatted Melanoma Patient Data form. The creation of this form led to a third and final review of the patient charts to obtain any additional information which had failed to be documented in the written data.

The computerized layout format for each patient (**Formatted Melanoma Patient Data**) (Appendix D) were compartmentalized into the following categories:

Patient information: date of birth, address, telephone number, Yale New Haven Hospital medical record unit number, surgical tissue pathology number, death date

Initial Diagnosis: date of initial diagnosis and year of initial diagnosis
Primary anatomical site
Primary Breslow thickness
Primary Clark Level
Primary Ulceration
Primary TIL
Primary Micro-satellitosis
Primary Regression

Stage at Initial Diagnosis	Restaging Date
T	T
N	N
M	M
LDH	L
ECOG Score	ECOG Score

Imaging Studies
CT scan
MRI
Chest x-ray
Pet scan

Pathology
First recurrence date
First recurrence site

First recurrence diagnosis mechanism
Second recurrence date
Second recurrence site
Second recurrence diagnosis mechanism
Third recurrence date
Third recurrence site
Third recurrence diagnosis mechanism

Treatment Information

Initial treatment start date
Initial treatment
Side effects from initial treatment
Initial treatment cause of discontinuation
Initial treatment discontinuation date

Subsequent treatment date
Subsequent treatment
Side effects from subsequent treatment
Subsequent treatment cause of discontinuation
Subsequent treatment discontinuation date

Oncotech Inc. information

Date of Oncotech Inc. report
Drug resistance assay
5FU:
alpha-IFN:
Bleomycin:
Carmustine:
Cisplatin:
Cisplatin +Gemcitabine:
Cyclophosphamide:
Dacarbazine:
Doxorubicin:
Etoposide:
Fluorouracil:
Gemcitabine:
Ifosfamide:
IL 2:
IL 2 + IFN:
Mitomycin C:
Navalbine:
Taxol:
Taxotere + Navelbine:
Taxotere:
Temozolomide:
Topotecan:
Vinblastine:

Results of Data from Yale Cancer Center Melanoma Weekly Conference

Additional information relevant to the study was obtained through weekly participation in the Yale Cancer Center Melanoma Unit Weekly Conference. The conference is held in private forum for the discussion of patients who are under the care of its members and is conducted under the direction of Dr.Ariyan. The weekly conference was begun primarily as a forum within which to present patient cases that are exhibiting poor outcomes and extreme drug resistance. The goal with these patients is to affect progress by contribution from group experience and expertise. The testing results

for Oncotech Laboratory Incorporated, as obtained for all melanoma patients presented in weekly conference treated by the surgeons and oncologists in the group were discussed individually and a new treatment plan was devised by the group. The data obtained from weekly participation in the Yale Cancer Center Melanoma Conference provided significant information relevant to this research as did interviews of its members during the two years.

The patients that were presented in conference for multi-disciplinary treatment consideration included those patients in whom standard treatment was initiated by the treating oncologist and in whom treatment was showing no success. The purpose of the multi-disciplinary approach is to collectively determine the course of plan based on review of the entire case and sequence of events with the group. The cases for that week are prepared in advance and all relevant imaging studies, pathology slides, and laboratory indices are made available for the group for review during that conference setting. Radiographs and pathology slides are brought to the conference room so that each member of the team may review the readings previously made by the radiologist and pathologists. The purpose of this aspect of the presentation is to ascertain any new conclusions the group may reach.

The pathologists review the findings from the slides while projected for group viewing. The radiologist reviews the clinical findings on the imaging projected for view by the group. The cases are discussed at length and various opinions are exchanged and as a result, a new treatment plan is developed. The presentation is documented by a medical secretary and the notes are included in the chart as are simultaneous notes taken by Dr. Ariyan.

Each week an average of three patients were presented at conference. A new treatment plan is established for each of the three patients presented in that session. The treatment plan is concurrently transcribed during the session for each individual patient and then transcribed into the medical record of that patient and is labeled as the new treatment plan developed by the Yale Cancer Center Melanoma Unit.

In conclusion, after the 18 month observation of 120 patients with stage III and IV malignant melanoma whose cases were presented and subsequent treatment plans devised by the Yale Cancer Center Melanoma Unit, a common theme which emerged is that these experts were cognizant of the specific concerns of the individual patient and this parameter was prominent in consideration of the revised treatment plan. This conclusion is consistent with previous studies of patients with psychosocial disorders which indicate that quality of life issues as perceived by the patient and viewed by the treating clinician is a very important determinant in developing the final treatment plan. It is conceivable that the same is true for patients with advanced stage melanoma. It should be noted that through the observations of the group, treating physicians continue to utilize eventiary medicine to develop the final treatment plan.

Surgical Component of Cohort Study

All patients in this study had been referred for surgical evaluation to Dr. Ariyan from either their primary care clinician for symptoms of melanoma or in most cases a dermatologist who had performed the surgical excisional biopsy yielding the initial diagnosis of primary cutaneous melanoma. Upon surgical consultation with Dr. Ariyan at the Yale Cancer Center Melanoma Unit, each patient in this study cohort underwent a full surgical procedure work-up according to protocol. Procedure protocol as set by Dr.

Ariyan is carried out on each patient referred to the Yale Cancer Center Melanoma Unit. Protocol included clinical evaluation to assess extent of disease. When indicated, the patient underwent further excision of cutaneous melanoma to include tumor margins, lymphoscintigraphy with SLN mapping and lymphadenectomy whether local or wide excision. Tumor tissue from each surgical procedure was provided for histology and staging as performed at A Yale New Haven pathology affiliate laboratory.

Each surgery was performed at Yale New Haven Hospital by Dr. Ariyan. A tumor tissue sample from each tumor and/or lymph node dissection was supplied as a fresh sample via overnight air freight to Oncotech Laboratory Incorporated in Tustin, California. Separate tumor tissue sample was additionally preserved on individual slides to be stored and catalogued in the pathology department at Yale Medical School in the laboratory of Dr. Rimm. These slides were subsequently used for a research study conducted at Yale Medical School. Seventeen of the 78 patients (22%) had more than one tumor tissue sample provided to the three laboratories within the years of 1994-2000 subsequent to their initial diagnosis of stage III or IV malignant melanoma. These additional samples over the course of their disease are due to progression of the disease.

Each patient was referred to Dr. Farber for the oncology management which continued for the extent of the patient's disease. Additional oncology consultations from other physicians contributed to a smaller percentage of patient care. Specialty consultation was obtained for the patients when clinical management of underlying systemic illness was warranted or toxic side effects extended to the domain of other medical disciplines. In such cases, written medical consultation notes were commonly provided to the surgeon and/or oncologist. Additional consultations or hospitalizations

unrelated to the melanoma resulted in changes to treatment in some cases. If these written consultations were not provided, the course of disease as seen in the medical charts might have been affected.

At the time of the diagnosis of malignant disease each patient was living in the northeastern region of the U. S. (Connecticut) with the exception of two patients, one lived part-time in Florida and another part-time in Dominican Republic. Relocation geographically during the course of disease occurred in a small portion of the patients. For some patients, seasonal migration from the northern states to the southern states had been routine prior to diagnosis, as was noted in the medical chart, and continued to a varying extent throughout the course of disease progression. Additional circumstances resulted in temporary or permanent moves in fewer patients. Examples are relocation to care for an offspring's newborn or assist with family illness or hardship. Some patients relocated temporarily to receive treatment or participate in clinical trials not available in Connecticut. An example of this was a temporary relocation by one patient to the Dominican Republic to obtain alternative treatment not approved in the United States in lieu of the recommended chemotherapy.

In cases when the patient relocated to participate in clinical trial or for personal reasons, medical care was continued under Drs. Ariyan and Farber. Geographic location whether temporary or permanent, was reflected in altered continuity of care, or for a few patients in the evaluative study, cessation of care and loss of follow-up data. Consultation transcriptions from outside clinicians were provided for the majority of patients in whom temporary or permanent relocation occurred. Nonetheless, changes in

the administration of medical care contributed to challenges in assessing the course of the disease for purposes of the research.

Prior to 1994 all but two patients in the study (97.5%) had documentation of cutaneous melanoma. Failing official documentation, 2.5% of patients either had prior cutaneous melanoma diagnosed without clinical documentation or had advanced disease without initial evidence of cutaneous disease. This statistic reflects the literature wherein five percent of patients have metastasis without evidence of prior cutaneous disease for the same reason (Cuevas and Whitman, 2002).

Frequency of Tumor Recurrence in Patients with Malignant Melanoma

Table 12 contains data about the age variables for the patients in the evaluative cohort. The average age of individuals for whom data was collected at the time of their initial diagnosis for melanoma was 56.9. The youngest person in the sample was 24 years old. The eldest was 90. The sample was disproportionately male, 47 of the 78 sample members. The males, on average, were younger than the females with respective average ages of 54.9 and 59.3.

The age at the time of first recurrence could only be calculated for individuals who were observed progressing to that point with the disease. There were 42 observations in the sample where age at this point could be calculated. The average age at first recurrence was 56.8 years old with a minimum of 38 and a maximum of 91.

Table 12 Cohort Sample Statistics

Mean Age		Mean(n)		Minimum Value	Maximum Value
Date Of Initial Diagnosis		56.9 (78)		24	90
At First Recurrence		56.8 (42)		38	91
Females		59.3 (31)		33	83
Males		54.9 (47)		24	90

Investigation of the Utilization of EDR Test Results

In order for the EDR test results conducted by Oncotech to be useful, they must be utilized. This section discusses information obtained through tabulations from medical records, attendance at weekly Melanoma Unit meetings, and discussions with Yale Cancer Center Medical Unit physicians regarding test utilization.

Dr. Ariyan provided tissue samples to Oncotech Inc. for testing from each surgical excision performed for the patients in this study. Results from tumor tissue sampling are provided to the treating surgeon and/or oncologist by Oncotech. Oncotech supplies a written **Drug Resistant Assay Report** (Appendix C) to the surgeon from each tissue sample tested by the EDR assay. Oncotech offers differential staining cytotoxicity and comprehensive immunohistochemistry testing on tissue samples. When requested, testing is conducted on samples and the results are provided in the **Prognostic and Predictive Marker Report** (report not shown) to the surgeon and/or oncologist. As is standard practice with the physicians at the Yale Cancer Center Melanoma Unit, both the treating oncologist and the surgeon have access to the report results. In 96% of the cases in this study the report was contained within the medical record in one of the named offices. In four of the cases, the report results were obtained from the office of Dr. Rimm, where they are also housed electronically for purposes of correlating data.

Neither the surgeon nor the oncologist is officially or legally obligated to utilize in vitro EDR test results. There is no guarantee that such results will be successfully translated from in vitro to in vivo models. In many cases the standards of these tests are determined by the companies and not be the U.S. Food and Drug Administration.

It was not possible to directly quantify utilization of the Oncotech test results as a determinant of treatment in absolute terms when analyzing the medical records. One reason for this is that in the overwhelming majority of cases individuals exhibited low or intermediate resistance to the drugs they were currently receiving. However, there were two cases in this study where an individual tested EDR for a drug they were receiving, and the physician discontinued that particular drug and started treatment with another. In both cases, written confirmation of the use of the Oncotech report in reaching that decision was obtained from the medical record. The decision to treat was based on Oncotech Inc. EDR testing results as well as determination that the disease was progressing as evidenced by imaging studies (chest radiograph and CT of the abdomen and pelvis) and clinical indicators (decreased ECOG scale) and increased toxic effects of medication. Through interviews with the treating physicians, confirmation was made to indicate that if the test results from the Oncotech EDR demonstrate EDR for the tumor tissue supplied for the individual patient, the physician does consider the results. However, other clinical factors enter into the final decision making including survival potential and the treatment plan as devised by the group at the Yale Cancer Center Melanoma Unit for those patients whose cases are reviewed.

Oncotech Inc. EDR Tissue Specimen Procedure

In addition to EDR in vitro test accuracy, turn-around time (evaluability rate) is of primary importance as a variable in the consideration of utilization of the test results as part of the treatment determination for the individual patient. The enhanced practical utility of in vitro testing was observed in learning of the development of the third generation assay technique used at Oncotech Inc. In comparison to the older clonogenic

systems which yield EDR test results in up to three weeks with 50% success rates, the newer techniques such as the one used at Oncotech conduct testing and provide turn-around time on average one week for the EDR assay (Appendix C) and three days for the angiogenic Prognostic/predictive marker results (Gliasite, 1993-2004).

For the study presented in this document, the evaluability rate for the Oncotech Inc. test results was within 85% of their stated turn-around time, which is similar to the turn-around time found in other studies reported in the literature (Mehta et al., 2001).

Comparison of EDR in vitro testing results turn-around time evaluability rate

The EDR results were provided by Oncotech Inc. to the ordering physicians and were located within the medical records of the patients. The results were received in a one week or less in 88% of the cases. Sixty-two patient assay results were reported within seven days. Three assay results were provided in eight days. The remaining eight cases exceeded three days with the maximum turn-around time being 15 days. According to the members of the Yale Cancer Center Melanoma Unit the short turn-around time for EDR testing results in this study was a favorable aspect of their services.

In addition to the EDR assay tests conducted on the samples of tumor from the 78 patients in the cohort study, 12 of the patient tumor tissue samples were also tested for angiogenic Prognostic/predictive markers. The evaluability rate for the Prognostic/predictive marker report for all 12 patients that had this test was greater than one week. Not all of the data supplied can be considered for utilization based on the reliability of the yield submission. As an example in one case, the results were indicative of insufficient sample thus full testing did not yield reliable results.

When results marked as insufficient data according to written provision by Oncotech Inc. it indicated that the specimen provided was not of sufficient size to perform the assay. Fourteen of the 100 samples tested at Oncotech for this cohort of 78 patients were identified on the EDR written report as having "insufficient data." However, 86% of the tumor specimens submitted yielded successful assays results. This finding is similar to the 90% accessibility rate that Oncotech Inc. reports from all samples they receive. This finding provides support for efficiency of testing results.

In vitro EDR assay result form

Oncotech provides correspondence to the physicians who use their service in the form of an order renewal request. The Assay Specimen Preparation Guideline (Appendix E) lists the various agents used by Oncotech Inc Laboratory for the tumor tissues tested. The renewal request solicits feedback from the physicians when ordering the testing panels for the specimen supplied to Oncotech with the option of customizing the panel. The ordering physician also has the option to request additional analysis for specific drugs to a given panel.

In the month of May in 2003, at Weekly Conference, the members of the Yale Cancer Center Melanoma Unit arranged additional meetings to formally discuss many aspects of the Oncotech data, its utilization, and to satisfy the order renewal request. The order renewal requests the physicians who order the EDR testing to select from two panels for use in future EDR testing for each individual specimen supplied to Oncotech Inc. Panel options include the Standard Drug Panel as constructed by Oncotech or the Custom Drug Panel as set by the ordering physician. Oncotech additionally offers that

specific individual requisitions for additional tests can be made for any specimen for an additional fee.

If one drug in a combination regimen goes untested, the results are less useful to the oncologist. The majority of the patients in this study were ultimately treated with multi-drug regimens: the Dartmouth regimen (dacarbazine, cisplatin, carmustine, and tamoxifen) or the CVD regimen (cisplatin, vinblastine, and dacarbazine) or with the addition of immunotherapy (alpha-interferon and/ or interleukin). For example, if IL-2, which is frequently part of the therapeutic regiment in the treatment of the patient with stage IV melanoma, is not tested for each tissue sample provided by the Yale physicians who order the EDR testing, then the value of the in vitro EDR test is diminished. This concern was expressed by the physicians in both interview and observation during Yale Cancer Center Melanoma Unit conference. The physicians in the group discussed the fact that the agents tested in standard and custom panels may not be sufficient or economic in their determination of extreme drug resistance. This possibility raises concern in utilization of the EDR testing given the addition of economic burden to the patient or insurer.

Validation of in vitro EDR assay Utilization

There were two cases where EDR testing from Oncotech Inc. Laboratory indicated extreme drug resistance to drugs being tested and that EDR was noted in the medical record. In addition to documentation of the EDR in these two cases by the physician, the drugs being administered were changed. This notation in the medical record by the treating physician indicates that the EDR in vitro testing results for these two patients augmented treatment decision. It is not entirely clear that the decision to

change the treatment regimen by the treating physician was made in exclusion of the other factors, but documentation of testing results and written notation of drug resistance to the drug being administered suggests inclusion of EDR test results.

This assertion was substantiated through interviews with the treating physician regarding these cases and regarding the overall consideration of the Oncotech Inc. EDR test results. Data collection was not conducted on specific points resulting from these interviews however was noted to substantiate qualitative findings during observations made for 18 months of weekly participation of the Yale Cancer Center Melanoma Unit Weekly Conference. The interviews took place in the offices of the respective physicians and as well at weekly conference.

The goals of the multi-disciplinary approach were provided for this research study presented in this document by the treating physicians who are members of the Yale Cancer Center Melanoma Unit during 18 months of participation in the conference where patients with malignant melanoma are presented for review by twelve experts in the field in order to devise alternate treatment plans and through individual interview. The goals discussed in conference and in individual interview with these experts established their approach. The approach taken is in concert with its membership and consideration of individual life issues, family concerns, employment, economics, comorbidity and personal choice was both known to the treating physicians and then shared amongst the members of the multi-disciplinary group as determination of the treatment plan was formulated in this forum for those patients for whom initial therapies appeared to fail. The general and clear assessment made was that the treating physicians in the Cancer Center Melanoma Group were astute to the individual concerns of the patient's quality of

life issues and such consideration was inclusive in the determination of the ultimate treatment plan.

Approach to Treatment Plan Determination Proceeds through Individual Case Presentation at the Yale Cancer Center Melanoma Unit

A typical individual case presentation involved the presentation of the pathology slide(s) from the patient's tumor(s) tissue projected for view by the team on a large scale projector. A Yale dermopathologists on the team presented the findings and participated in the group discussion. Likewise typically, a Yale radiologist on the team presented radiologic imaging studies performed during the course of disease or those relevant to current discussion and lead question and answer session by the team. A Yale geneticist and member of the Yale Cancer Center Melanoma Unit, expert in melanoma oncology research, provided expertise on a regular basis in these weekly conferences. A Yale pharmacology/oncologist, also a member of the group provided cutting edge expertise on treatment modalities for various phases of clinical trial study.

Several members of the team participate in numerous multi-center studies conducting clinical trials in addition to those conducted at Yale Medical School. This participation in multi-center studies allows the treating physicians to remain current with the latest treatments for advanced melanoma. To ensure that current information is garnered guest speakers are invited to participate in the group discussion every other month. For example Dr. D.A. Tuveson M.D. PhD., Assistant Professor, Departments of Medicine and Cancer Biology at the University of Pennsylvania, principal investigator Abrahamson Family Cancer Research Institute met with the group to discuss the status of Raf inhibitors in melanoma and other malignancies. Guest speakers in coming to Yale

Medical School to speak to faculty and staff were frequent guests who met with the Yale Cancer Center Melanoma Unit in the small group conference both to exchange shared information about developments in melanoma but also as a means to provide continuous interface with leading experts in clinical practice and research discovery from major cancer centers.

Relevant findings from evidence based medical trials and literature was presented at conference. A heuristic practice style using previous cases from the practice of the respective physicians as 'rules of thumb' was discussed during interview with the physicians. The treating physicians have several years of clinical experience treating patients with malignant melanoma and hundreds of patient cases from which to draw upon. The physicians that comprise the Yale Cancer Center Melanoma Unit have are well respected in the communities within which they practice, are well published and enjoy medical practice with large following.

Overall, the general practice style employed by the treating physicians collectively is a compilation of genuine compassion, 'rules of thumb' and knowledge of evidence based medicine. The practice style embodies the description of heuristic clinical practice with eloquence that is impressive and arguably becoming a lost art. Elements of uncertainty and complexity are integral to the many individual cases of advanced malignancies and melanoma is no exception. The multi-disciplinary approach employed by the physicians in the Yale Cancer Center Melanoma Group offers the inimitable balance of the advantages of existing clinical evidence and consideration of novel diagnostic testing with individual appointment of customized fit for a disease that at present is fraught with therapeutic challenge.

Despite the abundance of technology, clinicians still must use their judgment when making therapeutic decisions. The role of judgment and clinician expertise relative to the use of formal tests or evidence from research or clinical trials embodies the tension between what has been referred to as heuristic decision-making and evidence based medicine. Those clinicians who sustain integrity and ability to balance evidentiary medicine with lessons learned from experience while maintaining attention to personal story yield a very favorable outcome in their own right as providers of medical care to the individual and society.

The percentages of specimens that demonstrated IDR or LDR was very high with fewer drugs demonstrating EDR overall. While clear utilization or non-utilization is not known in all cases directly from the medical charts, the treatment continued without change based on the EDR testing from Oncotech. This result is consistent with continuing treatment with drugs for which extreme resistance has not been shown.

Investigation of Costs and the In Vitro Assay

The second hypothesis, whether in vitro tissue results are used by the clinician, their use will reduce costs and toxicity, will be discussed herein. Variables of cost which were identified as areas of concern for the treating physicians in the Yale Cancer Center Melanoma Group were individual cost of the test incurred by the patient or in some instances, the insurer, as well as additional costs incurred if the treating physicians requested additional drugs on the testing panel be conducted. One panel for EDR in vitro testing at Oncotech Inc. allows seven drugs to be tested under the base charge for the service. Beyond this, the physicians may order additional testing for an individual tumor tissue sample via an order renewal request which specifies that "additional tumor

morphometric analysis will be charged for more than seven drugs tested." Collectively the members in the group felt that the combination drug regimens they prescribe may not be consistently tested by choosing one exclusive panel as offered by Oncotech, Inc. The request that Oncotech Inc. test for additional drugs may generate additional cost to the patient. Given the frequency with which the drug regimen is modified for a given patient, indication of changes and therefore testing may prevent ideal testing inclusion.

The members of the Yale Cancer Center Melanoma Unit demonstrated and maintained consistent concern regarding the financial burden absorbed by their patients with malignant melanoma in all aspects of their practice. This concern for cost was both discussed in Weekly Conference and was noted in medical records. In some cases there were written exchanges between Dr. Ariyan and a given insurer where a cap was invoked or reimbursement for the in-patient surgical excision procedure at the hospital was declined. These were primarily cases where a patient had progressed to stage IV malignant melanoma and disease progress warranted surgical excision in the operating room for purposes of new staging and thus new treatment.

These written exchanges were located in the medical records as generated by Dr. Ariyan. When the written exchanges did occur, Dr. Ariyan provided a pedantic letter to the insurer detailing the progress and state of health of the patient and the need for surgery to remove advancing metastatic tumor. The letter sought reimbursement for the patient. Letters provided by the insurer in approximately 20% of cases declined the request and thus refusal of reimbursement. In instances of non-reimbursement Dr. Ariyan proceeded with the surgical procedure and absorbed the cost. This documentation was located in the medical records of the patients with whom it occurred. No patient was

denied care and in fact each patient in the evaluative cohort study and all of the patients discussed in the Yale Cancer Center Melanoma Unit, patients were offered continued care and surgery for progression irrespective of insurance reimbursement to the office practice or to Yale New Haven Hospital. To illustrate this, in four cases of 78 in the cohort study, the insurance company refused to reimburse for the surgical operating procedure to remove tumor tissue and surgical time to conduct the procedure by Dr. Ariyan. The documentation in the medical record indicates the exchange of letters between Dr. Ariyan and the insurer with details of the surgical procedure that ensued despite reimbursement. The explanation documented by the insurer all four cases were indicated as failure to obtain prior authorization by the patient for services rendered.

Five of 78 patients chose to terminate treatment or declined entry into clinical trials due to financial constraint as noted in the chart. The progression of disease in each of these patients continued through to death. Duration of course of illness for these five patients was not determined to be faster than others in the cohort. No patient in this study cohort who declined treatment or entry into clinical trials for experimental treatment demonstrated an increase in survival duration.

The cost of drug treatment to the patient for stage III malignant melanoma as of 2003 can average approximately $7000/month according to data pertinent to this study. Cost for treatment is solely based on estimate of chemo or biochemotherapy. The cost of drug treatment for stage IV disease with newer drugs including biochemotherapy can be in excess of $27, 000 per month. The absolute determination of average cost to the patient for drug treatment, resultant side effects and hospitalizations, in-patient operating room surgical procedure, medical office visits, and other associated costs as disease

progression occurs in malignant melanoma especially from stage III to stage IV was not possible from this study.

Discussion of Qualitative Issues of Cost in the Study: A Case Study

This study made clear the many challenges involving cost to the patient, to the physician and/or hospital and to society to treat this aggressive malignancy. There is no direct evidence for cost savings realized in this study. However, there is the possibility that the test results could be used in a manner which would lower costs. If a patient demonstrates LDR to a number of alternative drugs and if they each have equal treatment efficacy, it is reasonable to use the lowest cost alternative. Many of the patients in this study demonstrated LDR to several alternative drugs. This suggests one possible use for the EDR tests would be to construct reduced cost therapies. In cases where the test may indicate EDR to a current therapy, the impact on cost is not clear. This point can be demonstrated by considering the case of one patient that did demonstrate EDR to one of the drugs he was taking.

The patient was male and initially diagnosed at age 44 with stage II melanoma, Breslow depth 3.7, Clark level IV, 0 ulceration and 0 nodal involvement in 1995 for which he had surgical excision only to remove the cutaneous lesion. He was referred to Dr. Ariyan for surgical evaluation and received lymphoscintogram and lymphadenectomy to his left axilla and local wide excision (LWE) to the scalp all tissue pathology for melanoma was negative. Twenty-five months later 12/99 he developed dysplastic nevi to his left cutaneous scapular area which was removed via LWE by Dr. Ariyan. Tissue specimen was supplied to Oncotech Inc. He was started on Temozolomide and Thalidamide CCNU (Velban). The EDR test result was received and reported EDR to

the drug Velban. Dr. Farber made written notation in the medical chart as to the EDR to Velban seen in the Oncotech Inc. report. He noted that Velban would therefore be discontinued. Three months later the patient developed a cutaneous nodule on his back which was removed via LWE was advanced to stage III melanoma. Platinum was added to the drug regimen. The EDR tissue specimen had shown LDR to Platinum. The patient developed dizziness and rash suspected to be caused by the Temozolomide and was taken off this drug. The patient lived until September 2001 suggestive of a prolonged survival however unproven.

The discontinuation of Velban as a result of the EDR testing led to addition of the Platinum. The modification of the drug regimen after the EDR test results were reported may have avoided side effects that may have led to hospitalization that potentially would have contributed to a worsening of quality of life but certainly avoided the extra cost of side effects from a drug that has a large side effect profile and that would have cost a substantial amount of money for the cost of the drug.

Advantage to the Individual Patient by Multi-Disciplinary Approach When Considering Cost

While it is difficult to assess exact cost incurred and therefore cost savings analysis due to the varying individual needs and constraints realized in each individual case, there can be estimated benefits seen in the development of a cost analysis for a prospective patient who receives the individualized clinical approach employed by the multi-disciplinary group such as Yale Cancer Center Melanoma Unit. There are few published studies in the medical literature and of those, fewer are current, that have evaluated cost-effectiveness analysis of drugs specific to malignant melanoma. Messori

et al (1997) conducted a retrospective, incremental cost-effectiveness analysis on clinical data from a previously published ECOG trial. The Gompertz model was used to estimate the total lifetime values of patient-years of subjects receiving interferon in comparison to subjects given no adjuvant treatment. The ECOG trial involved 143 patients treated with high-dose IFN and 173 given no adjuvant treatment. Estimated drug expenditures were based on the assumption of a cost of $109.25 per 10 MU of IFN. The results of the pharmocoeconmic analysis showed that the adjuvant treatment of 100 subjects with high-dose IFN improved survival expectancy by 133.6 discounted life years or 308 undiscounted life years. The results from this study indicated that adjuvant treatment with high-dose IFN in patients with high-risk resected melanoma implied a favorable cost-effectiveness ratio. The Gompertz model analysis offers hypothetical results if a patient who develops advanced melanoma would live beyond the average of eight months. The study is not highly reproducible nor does it offer a standard from which to create cost-effective analysis for advanced melanoma.

Following the remainder of this section about to discuss the Yale Cancer Center Melanoma Unit and cost-effectiveness, the following section discusses quality of life as was included in the evaluation of the cohort group and those patient cases presented during conference. A distinct conclusion that is made resulting from the evaluations of both groups is that each individual case of the patients with stage III and particularly stage IV melanoma varies due to the economic burden imposed on the patient and/or family and the myriad of challenges that together can be categorized as quality of life issues that effect customized treatment plan for each patient. There is no uniform course of illness by which to group patients in a disease that takes life so quickly. Through

interview with the members of the Yale Cancer Center Melanoma Unit, through repeat review of medical records and through observation of the presentations of patient cases and subsequent treatment plan formulation, a pattern did develop to suggest that quality of life issues may be where emphasis of treatment planning must be directed to optimize patient choice.

The discussion from members of the Yale Cancer Center Melanoma Unit included frequent concern of economic burden in consideration of treatment. Costs and insurance reimbursement and drug effectiveness were consistent factors in discussion of the individual patient and their needs. In approximately 90% of the conferences during the time frame under observation for this study, a pharmaceutical representative was in attendance. The representative received no financial stipend for participation, yet the group allowed this participation in order to include updates to pharmaceutical research and pricing information. Calculations were made during meetings to establish the cost for a particular patient that was being discussed. This afforded calculations for ongoing treatment cost at the time of the discussion and thus treatment plan. On two occasions the members of the group chose to advise the patient to discontinue medication secondary to prohibitive cost and advised instead local excision of fast growing cutaneous lesions on the extremities. In the following two weeks for example, the patient choice was to undergo the procedure and the course of disease at time of study completion for these two patients demonstrated no worsening of symptoms (i.e. three months after surgical excision as evidenced by PET scan and CT of the abdomen and pelvis).

While there was no cost for the Yale Cancer Center Melanoma Unit conferencing, theoretically such cost if charged, for example for consultation services per hour per

patient, could be in excess of $1000 given that a dozen experts where involved on average. The benefit of a multi-disciplinary approach to treatment determination may be realized on an individual basis as suggested by findings in this study. The next section further discusses the varied role of quality of life issues for this disease.

Prevention of Toxicity

Reduction of toxicity is an important issue in case management. According to Smith and Bohurtha (1995), "the avoidance of unnecessary suffering, injury, or harm should be considered in oncology decision making." Oncotech Inc. claims that EDR testing saves patients unnecessary toxicity, saves valuable treatment time, and avoids the potential of inducing cross resistance to other effective agents.

Oncotech Assay Features and Benefits (Oncotech, 2003a)

Over 99% accuracy for identifying ineffective (resistant) agents
Independent of host factors
Avoids direct costs of ineffective therapies
Avoids costs of managing treatment related morbidity
Spares patients unnecessary toxicity
Saves valuable treatment time
Avoids the potential of inducing cross resistance to other effective agents
Approximately 90% of tumor specimens submitted yield successful assay results
Test results are available in 7 days

Oncotech Inc. portends in their literature that the EDR testing performed in their laboratory avoids direct costs of ineffective (resistant) therapies and avoids costs of managing treatment related morbidity. "Oncotech's integrating drug development services provides information and insight to enable innovative pharmaceutical companies to conduct smaller more focused and successful clinical trials, thereby reducing the costs and time required to gain FDA approval and move product to market." (Oncotech, 2003b).

Toxicity secondary to chemotherapeutic agents are of substantial significance to the patient and for the oncologist. However a recent study showed that compared to non-cancer controls, women with recurrent breast cancer overwhelmingly preferred salvage therapy to palliation. Quality of life was of secondary importance to the desire to continue aggressive treatment. The heterogeneity of response to treatment in recurrent ovarian cancer makes choice of treatment difficult (Sharma et al., 2003). There is no study from the medical literature evaluating salvage therapy preference over palliation with malignant melanoma however palliation is often the most realistic goal in treatment in stage IV melanoma. The treatment for malignant melanoma is fraught with a robust side effect profile which can greatly affect quality of life, yet the need to treat aggressively to combat its natural progress is warranted.

The patients in this cohort study and the observational conference patients experienced a myriad of side effects paralleling the side effect profile seen in general in malignant melanoma. Grade 1 and 2 toxicities, including fever, chills, nausea, skin rashes, anemia, neutropenia, and thrombocytopenia, were seen in most patients in this study regardless of the specific drug regimen. The incidences of grade 3 or 4 toxicities including hypotension, nausea and vomiting, thrombocytopenia, neutropenia, and cardiac toxicity was seen in many patients receiving immunotherapy (Appendix D) There were patients who received off-protocol treatment who experienced side effects ranging from mild to severe. Those with severe side effects required hospitalization. Side effects and hospital course were obtained from the medical records and translated to the **Concise Tabulated Melanoma EDR Data** (Appendix B).

Several factors contribute to the quality of life for a patient. Overall health and ability to care for self, family, and hold a job are major factors that affect the quality of life. The ECOG score is used to evaluate and quantify quality of life in the patient and was recorded in the medical charts of the patients with varied consistency. The ECOG score was rarely recorded at the initial diagnosis however became utilized with fair consistency at the development of recurrence in 30% of the cases or 23 patients (Appendix B).

Overall an ECOG score was recorded in the medical chart at least once in 70% of cases or 55 patients. The documentation of the ECOG score was recorded most often in relation to the onset of side effects in relation to administration of therapeutic drugs. In 55% of the cases or 43 patients, there were two recordings of the ECOG score at onset of side effect and after dose reduction or discontinuation. In 88% of the cases the dose reduction or discontinuation of drug(s) resulted in an improvement of the ECOG score.

This study is reflective of other studies that report the varied side effect profile of the chemotherapies and immunotherapies administered for malignant melanoma. Disease progression was seen with more frequency than side effects were recorded. The modification to drug regimen was more reflective of this factor than of side effects. When it was documented in the medical chart, the oncologist and the patient continued the drug(s) with a dose reduction over discontinuation. In three cases, documentation indicates the discontinuation of drug(s) secondary to side effects or toxicity. These were nausea and weight loss, severe intolerable headache in a patient who was CT-scan and MRI negative for brain metastases with severe depression, and the third patient had neutropenia and vertigo. There were 30% of cases where clear documentation is made

indicating untoward side effects, both grade 2 and 3 all in whom the dose was halved or temporarily discontinued. In these cases the drug was re-administered at half dose with a gradual increase or left at half-dose wherein side effects were diminished or tolerated better.

Most changes to the drug regimen in this study were made with progression of disease and recurrence. Drug(s) were discontinued is cases where the onset of recurrence was generally within two months of administration of drug(s) and in such cases new therapy was administered. When it became clear that patients were progressing rapidly preventative efforts by changing therapy for example change in therapy was made in patients with ongoing systemic response to biochemotherapy by modifying the biochemotherapy regimen by replacing dacarbazine (DTIC) with oral temozolamide.

Many of the patients in this study, 73% (59 patients) were prescribed interferon-alpha or Melphalan through protocol at some point in the course of their illness. The typical side effects seen with the administration of interferon were documented in the medical charts. Many of the patients had mild to extreme nausea, weight loss, and/or depression. Aggressive antiemetics were provided for nausea and referral to psychotherapy was common in a fair number of patients. Fifteen percent of the cases or 12 patients were on anti-depressants and/or benzodiazepine to augment symptoms. Despite symptoms, the majority of patients remained on interferon whether reduced dose or full dose through to completion of treatment.

As patients did progress IL-2 was added to the drug regimen. The EDR test results from Oncotech Inc. may have influenced the oncologist to make changes to the drug regimen based on side effects or toxicity but such influence could not be confirmed

through chart documentation. If a patient exhibited intolerable side effects or toxicity resulted in severe systemic harm, the patient was taken off the drug thought to cause the reaction. Although they pose the greatest symptoms regarding toxicity, IL-2 and interferon were tested very infrequently in the tissue samples from the patients in this cohort. The combination of IL-2 and IL-2 +interferon was tested six times. Interluekin-2 alone was tested once. Interferon was tested five times. Only in two cases were all three tested together. In all cases where IL-2 and IL-2 + interferon were tested, the results from Oncotech Inc were reported as EDR but the side effects seen in the patients did not warrant discontinuation of drug. The life span for the patients with stage IV malignant melanoma limits what can be observed insofar as Oncotech Inc. results.

This study exemplified related clinical challenges related to treating the patient with malignant melanoma. There were a range of single and multiple underlying medical and psychological illnesses that predated the onset of melanoma or recurrence in the patients in this study including hypertension, COPD, dyspepsia, alcoholism and depression for example. In general, patients are in beyond the forth decade of life when diagnosed with malignant melanoma. In the cohort group the mean age was 41 years of age. Underlying state of health at time of diagnosis and subsequent exacerbation of underlying disease and new onset systemic illness in association with melanoma malignancy may increase complications of the state of health for the individual patient.

Progression of the disease resulted in systemic symptoms including the central nervous system from brain metastases that caused attendant vertigo or headache, hepatic metastases causing abdominal pain and nausea, hemorrhage and GI bleed from colon,

soft tissue injuries due to incidental trauma, and a range of pulmonary changes as consequence to lung metastasis. No patient in this study was HIV positive.

Social and psychological behavior can have a tremendous impact on perception of the patient in disease improvement or failure, compliance with medication adherence, scheduling and reporting to the physician, scheduling of medical appointments and clinical trial participation, and ability to cope with the many effects of a disease that often ends in rapid demise. Tobacco use was not entered as a variable in this study once it was determined that smoking history was not documented in the medical charts in all cases. However where it was documented, it was calculated that 40% of the patients in the study had a positive history individually of approximately 20-40 pack years. Tobacco use is associated with being causative of certain cancers and is also associated with poor systemic health. Alcohol abuse in this study affected one patient in particular who attended in-patient rehabilitation programs throughout the course of her illness. Her relapse into alcoholism during the progression of illness, stage III melanoma, led to missed appointments (by her admission as documented in her medical record) and ultimately to her stopping the drug regimen that was prescribed.

Depression secondary to interferon in doses administered for malignant melanoma was seen in the study in approximately 30% of the patients in the study. This finding was consistent with this reported side effect of the drug as noted in this document. This factor alone involved an increase in economic burden to the patient, alteration in patient compliance to medication and in approximately 25% of the patients, interferon had to be reduced or discontinued to this side effect alone. For those patients where this was noted in the medical record of the patient in the cohort and in those patients

discussed at Melanoma conference, a range of time intervals was found for restart of interferon or temporary or complete discontinuation of interferon and replacement with alternative trial with another agent was attempted. In the conference setting this issues are salient and discussed with earnest by the treating physician group. The variation in administration of interferon within the cohort group in response to social and psychological affects for the individual patients gives credence to the notion that there is not a clear standard regimen that works for all patients with malignant melanoma.

The physicians in the multi-disciplinary group discussed issues pertaining to individual patients, including family dynamics, travel concerns, employment disruption, family wishes, disease denial on the part of the patient, congenital mental impairment, morbid obesity preventing ideal surgical treatment and a whole host of individual patient concerns which the group as a whole, and independently, the members embraced to provide optimal attention for each case.

Recent data on single nucleotides has also provided new insight into the genetic basis for patient toxicity whereby a fraction of patients are shown to rapidly inactivate drugs, while others suffer greater toxicity by virtue of slower drug metabolism (Roses 2001). Individual differences in drug metabolism that might prevent an active form of the drug from reaching the tumor in vivo cannot be modeled against the current in vitro assay systems. Many variables contribute to the challenge of treating the patients with malignant melanoma. The claims that Oncotech Inc. makes about the humane benefits to EDR testing have been evaluated through the studies with breast and ovarian cancer and have shown promise and positive outcomes. This study does not replicate those findings; however, it is possible that future studies evaluating malignant melanoma and Oncotech

Inc. EDR testing may show the same promise and outcomes. The hypothesis suggested that if in vitro tissue results are used by the clinician, the patient will benefit through decreased toxicity, was not proven in this study.

Discussion of qualitative findings from the Yale Cancer Center Melanoma Unit

Based on the observations made from the conference and the interviews with the individual experts that comprise the Melanoma Unit, the multi-disciplinary approach is of benefit to their practice and the satisfaction to the patient.

Determining a treatment plan for the patient who has already demonstrated drug resistance of their tumor as supported by EDR testing results from Oncotech and by ongoing imaging studies and clinical evidence is difficult as was stated throughout the duration of the study by the treating physicians. The treating physicians all agreed that the contribution of the group members with their collected expertise (oncology, general and plastic surgery, radiology, dermopathology, pharmacology and epidemiology) offers a synergistic outcome which can be found to be of benefit to the patient, patient's family and the treating physician in consideration of the individual patient needs.

According to each member of the Melanoma Unit, continued trial in utilization of Oncotech EDR data for malignant melanoma is warranted for the list of EDR assay features and benefits as suggested by Oncotech Inc.: test accuracy in identification of ineffective agents, avoidance of direct and indirect costs of ineffective therapies and cost of managing treatment related to morbidity, sparing unnecessary toxicity, saving treatment time, avoiding potential cross resistance and success of test result turn-around time. The interviews conducted resulted in overall favor of continued consideration of utilization of the Oncotech Inc EDR testing for malignant melanoma.

Effects on Survival Rate

The third hypothesis of the research is that if in vitro tissue results are used by the clinician, the patient will benefit through increased progression to survival. While it is clear that in vitro drug-response assays effectively discriminate between clinically inactive and active agents, this does not necessarily translate to an accurate prediction of patient survival. Various clinical trials have identified agents capable of causing short-term responses without translating clinical response into survival benefit. Clinical validation of in vitro drug-resistance assays requires that they predict poorer survival for patients treated with agents their tumors are resistant to in vitro and improved survival for patients treated with agents their tumors are sensitive to in vitro.

Oncotech claims that patients receiving chemotherapy drugs that were found to be in the extreme drug resistance category for their tumor had significantly shorter disease-free and overall survival rates based on previous recent studies (Mehta et al., 2001). There does not appear to be strong evidence that patients treated with an EDR-assay directed therapy would have received different drugs than the ones they did receive in the clinical setting of this study. The reason for this is that in the vast majority of cases patients were receiving drugs to which they demonstrated LDR. This allows a contrast to be made between those who received therapies which would be consistent with an assay directed therapy to those who tested EDR to their current therapy.

In vitro patterns of resistance varied among patients, with few patients showing resistance to all drugs tested. This suggests that alternative drugs may have been available to choose from for patients when one specific drug was found to be inactive in vitro for a given patient.

The clinical value of in vitro predictive tests is based on the fact that treatment outcomes for malignant melanoma are still unsatisfactory. Most patients with stage IV malignant melanoma eventually die of recurrent, drug resistant cancer. Multi-agent therapy is an important component of treatment. Biochemotherapy (chemotherapy + immunotherapy) with alpha-interferon and especially IL-2 are used commonly in advanced malignant melanoma as seen in this study. Interleukin-2 is costly and has a very high side-effect profile requiring hospitalization for administration. Seven of the specimens that were tested for EDR in this study demonstrated EDR to IL-2. Each of the seven patients was on a biochemotherapy regimen. Because some patients in general with malignant melanoma appear to respond to IL-2 therapy, patients in this study who were on a multi-drug therapy were kept on IL-2. Clinical drug resistance is not an all-or-none phenomenon; therefore, patients do continue the drug with the caveat that those patients whose tumors do demonstrate EDR to IL-2 may be at high risk for early progression and may be candidates for novel agents and/ or combinations in the protocol setting.

Survival is typically shorter in patients with melanoma who relapse after responses to biochemotherapy. Five patients of 78 in this study initially demonstrated a regression of their tumors as seen by CT scan, MRI, or Pet scan who then went on to a fairly rapid progression of their disease. Several small studies have indicated that

response rates are improved when patients received chemotherapy to which their tumors were not resistant in vitro (Freuhauf and Bosanquet, 1993). In the evaluative cohort all but four patients received drugs to which they were not resistant. The implication is that longer intervals to progression and/or increases survival times would be expected in this cohort of patients. Alternatively, shorter intervals to progression and reduced survivals would be expected for the group of patients receiving therapies to which their tests indicated EDR.

The drug exposures used in the EDR assay are 5-10 times higher than those achieved in vivo, biasing assay reliability towards accurate detection of drug resistance. The prediction of resistance may be more robust than the prediction of sensitivity because of the inability of in vitro systems to parallel relevant in vivo pharmacodynamics, such as individual variations in tumor vascular supply and drug metabolism. The relationship between in vitro results and survival has not been adequately addressed in either chemosensitivity or chemoresistance assays.

A pattern observed in this study was shorter intervals of progression of disease and survival time in patients who demonstrated EDR on in vitro test results to their current drugs. The average patient in this study had a 19.8 month interval between the initial diagnosis of primary tumor (prior to surgical referral to Dr. Ariyan) and the first recurrence. For those patients who tested EDR to their current therapy the same interval was only 9.8 months. Similarly, for the average patient in this study, the interval between the first and second recurrence was 9.0 months. For those who tested EDR to their current therapy, that same interval was only 4.3 months. These three observations for a sample of patients with malignant melanoma are consistent with other studies for ovarian

and breast cancer which have shown that patients who test EDR to the drugs they are currently receiving are observed to have more rapid disease progression.

A similar observation can be drawn for the patients in the study with malignant melanoma regarding EDR and survival times. The average survival time from the initial diagnosis of the primary melanoma was 41.3 months or three and one half years. For those patients who tested EDR to at least one of the drugs in their current therapy, the average survival interval beyond initial diagnosis was 25.8 months. Again this pattern is consistent with the broader medical literature regarding EDR testing in ovarian and breast cancer. There it was observed that patients who tested EDR to drugs that were part of their current therapeutic regimen experienced shorter survival times, Table 13.

Table 13 Times to Progression and Survival Time in Months

Category:		First Recurrence		Second Recurrence		Months to Death	
		Mean (Std. Dev)	Median (N)	Mean (Std. Dev.)	Median (N)	Mean (Std. Dev.)	Median (N)
Entire Sample:		19.8 (23.7)	9.0 (41)	9.0 (8.9)	5.5 (28)	41.3 (36.9)	29.5 (26)
Sex:							
	Female	24.3 (31.2)	9.0 (17)	7.1 (5.7)	4.5 (12)	51.2 (45.5)	31.5 (12)
	Male	16.7 (16.5)	9.0 (24)	10.5 (10.6)	6.5 (16)	32.8 (26.5)	26.5 (14)
Age:							
	≤50	23.7 (28.1)	9.5 (16)	10.8 (9.3)	8.5 (12)	36.9 (28.5)	32 .0(11)
	>50	17.4 (20.6)	9.0 (25)	7.7 (8.6)	4.0 (16)	44.5 (42.7)	28.0 (15)
Staging of Primary:							
	I	22.8 (28.3)	13.5 (4)	6.5 (9.0)	2.5 (4)	42.7 (36.3)	28.0 (3)
	II	11.0 (9.7)	8.0 (4)	13.0 (---)	13.0 (1)	22 .0(12.1)	15.0 (3)
	III	17.5 (27.0)	7.0 (15)	9.0 (10.3)	4.5 (12)	31.2 (25.7)	10.0 (10)
	IV	17.0 (12.1)	24.0 (3)	30 (----)	30.0 (1)	89.0 (7.1)	89.0 (2)
EDR for Any Tested Drug:							
	No	17.5 (17.8)	7.0 (23)	8.1 (7.8)	5.0 (15)	42.0 (30.8)	31.5 (12)
	Yes	22.6 (27.9)	9.0 (23)	10.1 (10.0)	7.0 (13)	40.6 (42.6)	23.5 (14)
EDR to Current Therapy:		9.8 (10.7)	6.5 (4)	4.3 (3.5)	4.0 (3)	25.8 (7.1)	23.5 (4)

Table 13 above tabulates the intervals to recurrence as well as survival times. In addition to the patterns discussed in the text, separate calculations are provided for the following groupings; gender, age, staging of primary melanoma at initial diagnosis, and whether a specimen demonstrated EDR for any drug tested. Median values for each of these statistics are also reported in Table 13. Using the medians the same patterns noted for the means are observed although they are less pronounced.

This study reproduced findings in previous studies that demonstrate the multiple drug resistance patterns seen in the tumors of metastatic cancer as it progresses. The collective number of cytotoxic drugs studied in my research was twenty-two. Over a period of eight years (1994-2000), Oncotech tested eighteen of these possible 22 agents, and fourteen of these drugs demonstrated EDR in at least one tissue sample. The average number of cytotoxic agents tested for any given specimen was 7.8. In 36.5 percent of tissue samples tested (n=85), EDR was observed to at least one of the drugs tested; 20 percent of tissues showed EDR to three or more drugs tested; and 3.5 percent of tissues showed EDR to five or more drugs tested.

There is no chemotherapeutic agent that has been proven to significantly increase survival rates in metastatic melanoma in recent literature or practice. This study did not prove or disprove the hypothesis that utilization of Oncotech data will increase survival. However, evidence was provided that may suggest that patients who exhibited EDR to their current therapy experience more rapid progression of their disease and shorter mean survival times. This finding suggests that further testing of the utilization of EDR in vitro tests may guide the treating physician from agents that are found to be resistant in vitro.

This finding may ultimately lead to use of alternate agents, discontinuation of an agent demonstrating in vitro resistance or may direct the treating physician away from the agent in consideration of accompanying side effects deemed to alter desired quality of life by the individual patient.

Heuristic Approach in the Yale Cancer Center Melanoma Unit

Although the research focused on specific hypotheses as previously discussed, the study as a whole provides evidence about the effectiveness of a heurisitically-guided clinical practice relative to hypothetical treatments that might have been given under an assay-directed therapy. In the academic literature there are studies (cited henceforth) wherein the authors have written as though clinical rules of thumb are always divergent from evidenced-based practice. However, others have noted that the evolution of rules of thumb is one method of deriving an evidenced-based rule. The evidence in this study is consistent with this latter proposition: the clinical heuristic developed at the Yale Cancer Center Melanoma Unit resulted in treatments consistent with the results from ex post tests.

There were 78 patients in the cohort study. In all cases, the treatment regimes of patients were established prior to the test being performed. Only four patients subsequently showed extreme drug resistance to the drug tested. This suggests that the clinical heuristic developed within the Yale Cancer Center Melanoma Unit led to treatment decisions that were overwhelmingly consistent with those that would have been indicated through an assay-directed therapy.

Scholarly research has developed alternative theories of the evolution of heuristical practice rules. The application of the major elements of at least one of these

theories could readily be seen in the decision making of the Yale Cancer Center Melanoma Unit as it related to therapeutic determination and ongoing evolution of the practice by the surgeons and oncologists in this study.

Many hold the view that research is solely the province of educational institutions and that case practice is a fundamentally different activity; however, according to Poulter (2003), there are two distinct but intertwined forms of research seamlessly embedded into clinical practice, interventive research and modeling research. Interventive research is aimed at developing the "theory of one," this refers to the process of understanding an individual patient's circumstances in order to formulate appropriate individualized interventions (Poulter, 2003). Modeling research refers to a synthetic process which links individual practice insights gained from many cases of one class observed during interventive research.

According to Pouter's theory, these areas of research entail two distinct branches of activity. In interventive research, cases are treated reflexively. This refers to the activities of observing, describing, abstracting, generalizing, assimilating, and acting. In modeling research, the information obtained from the interventive level is woven into a process of analytical induction involving the additional activities of categorizing, ordering, contextualizing, modeling, and accommodation (Berlyn, 1957; Poulter, 2003). The development of a heuristic rule has occurred when inductive insights are reached that abridge the reflective process as new cases present themselves. Thus, the process results in new knowledge by establishing or postulating relationships between insights gained in a class of related cases.

The model, as described by Poulter (Poulter, 2003), is taken from a retrospective viewpoint of what usually occurs in practice. The immediate requirements of daily practice can force one into the reflexive activity; nonetheless, a duality can exist in daily practice as the clinician reflects on the relationship of an individual case to others and assimilates new information from the current case into their store of knowledge. The relationship between the reflexive and reflective elements of practice and thought are likely to occur in a fluid relationship to each other rather than within a rigidly linear sequential thought process. New rules that guide reflective activities as new cases present themselves are obtained in this manner (Poulter, 2003).

It has been observed and affirmed by members in the Yale Cancer Center Melanoma Unit that their practice style is one of heuristic nature. Clinical decisions for treatment were almost universally consistent with EDR test results that arrived later. It could be argued that EDR test results should be used to perform assay directed therapies where appropriate. Ideally then the EDR test results would precede drug treatment determination. Ideally the drug choice would be reflected based upon whether the tumor tissue exhibited EDR, IDR or LDR. As it turns out despite the fact that therapies were initiated prior to the receipt of EDR test results in this study, they were rarely in conflict with the test results that arrived initiation of treatment. This observation supports the notion of an effective heuristic for the clinical practice of the Yale Cancer Center Melanoma Unit. To demonstrate this, a discussion will follow depicting how elements of that practice may be viewed through the lens of Poulter's theory.

The surgeons and oncologist who treated the patients in the study incorporated each step of interventive and modeling research into their clinical practice. The Yale

Cancer Center Melanoma Unit is under the direction of Dr. Steven Ariyan. Under his direction, the clinical style of practice is conducted in a very consistent, reflexive manner for the treatment phase. Interventive research was conducted for each patient through information gathering and assessment. Each of the 78 patients was given careful consideration regarding the complexity of their individual circumstance. One excellent indication of specific attention given to each individual case was seen in the extremely thorough charting of patient needs particular to and prior to the initiation of treatment.

The files for each of the 78 patients in the study had thorough documentation regarding their particular needs. Concerns were noted about interruption of employment, difficulties involved in relocation for clinical trial participation, familial well-being in the absence of the patient, complexities related to in-patient treatment administration, and ongoing simultaneous medical concerns for the patient outside the expertise of the specialty (e.g. many patients required counseling for the side effect of depression common as a side effect of the drugs as well as the issues surrounding diagnosis). One patient in the cohort study traveled to the Dominican Republic to obtain alternative naturopathic medication. Instructions for the medication were provided by the prescribing physician to Dr. Ariyan. The patient preferred taking this medication as well as treatment by the physicians in the Yale Cancer Center Melanoma Unit in effort to exhaust all possible curative measures. The physicians in the Dominican Republic and at Yale were in compliance.

Treatment for advanced melanoma can exceed $20,000 per month for the experimental therapeutics. While the treating physicians cannot affect cost structure, their practice consistently attempts to consider alternate options as well as clinical

treatment and counsel without charge. Issues relating to economic burden of treatment were disclosed in the data from the evaluative cohort and observations made from the Yale Cancer Center Melanoma Weekly Conference discussions.

Once a patient is referred to the Yale Cancer Center Melanoma Unit and diagnosis of metastasis is confirmed by histology from the tumor tissue surgically removed by Dr. Ariyan, treatment is initiated by the treating oncologist in the group. Challenging factors specific to the individual patient are taken into consideration for treatment determination such as comorbities, age, economic burden, familial issues, patient preference, staging of illness and additional concerns. Thus social context relevant to each patient, the state of the disease as it was diagnosed at the time, and quality of life issues specific to the individual patient are brought up for the patient. In total, it is accurate to say that the practice can clearly be seen to have incorporated many of the steps of what Poulter (2003) called interventive research which consisted of the elements of observing, describing, abstracting, generalizing, and acting to formulate a therapeutic plan.

A large component of the development of heuristic style is reflection. Each physician in the group would be expected as a normal course of activity to reflect on their own clinical experiences and modify their practice in the direction of therapies that lead to more favorable outcomes. In the group, the process of collective reflection was seen most clearly in the weekly meetings of the Yale Cancer Center Melanoma Unit.

The weekly meetings of the Yale Cancer Center Melanoma Unit were developed explicitly to serve as a forum for cases where the primary treating physician wanted the input of other clinicians. A typical presentation would include relevant clinical detail

along with slides of both the cancer site and cells. The audience consisted of clinicians and researchers in the area. Questions during the sessions were probative, searching for additional avenues of treatment. The meetings called upon the existing store of knowledge from the assembled group but also provided for the opportunity to observe anomalously difficult cases. These exceptional cases provided the group with the opportunity to see variation in disease progress and to formulate new thoughts regarding treatment decisions or unresolved clinical dilemmas.

Many of the members of the Yale Cancer Center Melanoma Unit also contributed to clinical trial development and progress. As part of their practices, they collected data and outcomes to provide to the investigators to help further knowledge regarding outcomes and drug response. Through trial participation and mutual exchange of data, the members of the group stayed knowledgeable regarding current clinical trials. There was no financial reimbursement for the members of the group for the time spent on data collection or clinical trial participation. Rather, this was a sign of the commitment of the group towards finding more effective therapies. The group discussed the state of clinical trials on a regular basis in the Melanoma Unit Weekly conference. The members of the groups were apprised of the details of clinical trials available, the content, avenues necessary to refer their patients when appropriate and the current state of each trial's status. The information was developed partially through their own practices. Also, the incorporation of knowledge about clinical trials and their results had a direct impact on the reflexive component of their practices.

The weekly meetings also resulted in modification over time of the physical environment in which the group practiced in order to facilitate a more integrated

treatment setting for patients. By integrating the necessary clinical components for treatment of melanoma, it was thought that better management of the disease would be possible with less stress placed on patients.

Thus, the group spear-headed the development of a treatment floor equipped with necessary staff (physicians and nursing) at Yale New Haven Hospital so their patients could receive the latest treatments. The surgeons and oncologist in the Yale Cancer Center Melanoma Unit was proficient regarding compliance issues, technical advancement, and other issues related to establishing this section of the hospital to help their patients avoid the complication of alternate locations for treatment that needed intravenous administration and nursing attention with physician back-up as warranted. This way, treatment and medication side effects could be witnessed and recorded in the same facility.

Relevant industrial representatives were also invited at times to the weekly meetings. Test results from Oncotech Inc. were often discussed at the Weekly conference. Relevant to these discussions, the director of Oncotech, Dr. John Fruehauf came to meet with the group and discuss utilization of the in vitro assays for melanoma. This effort provided an opportunity for face-to-face interaction which yielded clarification about the suggestions from the group that the test panel become more consistent with common treatment regimens employed by those in the group. Collective communication with Oncotech Inc. provided an opportunity for clinicians to clarify the appropriate interpretation of existing test results and to offer feedback to the firm regarding what would be more useful within their practice.

Individual clinicians reflect on their own experiences and update their practice styles, it was clear that the weekly meetings of the Yale Cancer Center Melanoma Unit served a similar function for the group as a whole. Difficult cases were discussed. The knowledge of other experts was available in this setting to formulate a better treatment. The case could be assimilated and categorized for future reference. Everyone present had the opportunity to enrich their current base of knowledge by being exposed to the thoughts of the larger group. New models for treatment were established through this interactive exchange.

In addition, new ideas were brought into the group through ongoing research and clinical trials. Again, this allowed members of the group to place their own cases in the context of an overlaying paradigm. This process calls for categorization and assimilation of knowledge as described by Poulter.

As concerns were raised regarding the practice, the group assimilated that knowledge and acted to improve the facilities available. When deemed useful, outside representatives from industry were invited to attend conference. The purpose of such representation was ongoing exchange of information that could be integrated into the practice style which ultimately impacted the reflexive style for dealing with patients.

Conclusion to Chapter 3

While matching a specific clinical experience with a theory will never be a perfect fit, it is clear that the operation of the Yale Cancer Center Melanoma Group largely consistent with the manner in which Poulter (2003) describes the development of a reflexive heuristic style which evolves over time through either individual or group reflection. Based on the evidence reviewed in this study, the Yale Cancer Center

Melanoma Group developed a highly effective practice through a process of heuristic learning. One indicator of the effectiveness of the practice is the prescription of treatment regimens for patients prior to testing for drug resistance seldom found to be in conflict with those test results.

This study set out to evaluate utilization of in vitro testing within the medical community for advanced melanoma. In patients who progress to advanced melanoma, only low response rates (between 10-15%) have been achieved by the most effective cytostatics in single-agent therapy, leading to a mean five--year survival in less than five percent of patients (Prignano et al,: 2002). While the survival rates of patients with the cutaneous form of melanoma have improved greatly over the past decade as a result of owing largely toearly diagnosis, scant the alignant form of the inverse is true of the malignant advanced form of melanoma (Prignano et al,: 2002). Chapter 4 discusses the heuristics and decision making in light of therapeutic decision making. The final chapter, Chapter 5, will provide a discussion about new approaches in the treatment of malignant melanoma. The chapter also includes a proposed model for a melanoma unit using the elements and data defined in this study. The chapter and documents ends with conclusions about physician approach to a disease which at present offers distinct challenge in therapeutics most primarily as a result of extreme drug resistance to existing therapies.

CHAPTER 4: HEURISTIC CLINICAL APPROACH, EVIDENCE BASED MEDICINE AND MALIGNANT MELANOMA

The research presented in the next chapter considers the practice of the Yale Cancer Center Melanoma Unit. Within their practice, a style of treatment has developed within which, the disease is treated upon receipt of the pathology report which allows staging of the disease. The primary consideration in this approach in the cases being examined is the rapid progression to death given all of the patients in the study are diagnosed with Stage III or IV malignant melanoma.

At the time of tumor excision for the patients, a tissue sample is sent to Oncotech Inc. for EDR testing. Those results are not available prior to the initiation of treatment. In the next chapter, some of the standard outcomes seen in the literature for EDR tests will be examined such as time to survival for different levels of resistance. Another relevant issue is whether the local practice style of the Yale Cancer Center Melanoma Unit leads to treatments that are consistent with the EDR test results.

The EDR test results for melanoma are still considered experimental in nature by the FDA and thus need not be considered by any physician as part of a diagnosis or establishment of a treatment. Tumor tissue from surgically removed malignancies of advanced melanoma is being provided by some of the surgeons who comprise the Yale Cancer Center Melanoma Unit for in vitro EDR testing at Oncotech Inc. Laboratory. The results from the in vitro assays as provided by Oncotech Inc. Laboratory, is being considered to augment treatment decision making for the same physicians who treat the disease. The role of the in vitro testing is considered as a potential economic benefit to the patient and general medical environment. Taking into account the benefits under

study in vitro assessment for other solid malignancies (Villman et al., 2005) the consideration given by the treating physicians at Yale Cancer Center Melanoma Unit is well founded.

To examine the predictive value of a short-term in vitro total cell kill assay, 37 patients with breast cancer were studied in Sweden. Tumor cells were prepared from tumor samples from 17 patients with locally advanced and 20 with metastatic breast cancer, which were treated with FEC (5-flurouracil, epirubicin and cyclophosphamide) regimen or a combination of epirubicin and taxanes. The cells were tested using a fluorometric micro-culture cytotoxicity assay (FMCA), which is based on the conversion by viable cells of fluorescein diacetate to fluorescent fluorescein, for sensitivity to the drugs given in vivo. The FMCA data were scored as low, intermediate or extreme drug resistance based on the median survival +/- SD for each drug and patient subset. The drug classification for each sample was then correlated to clinical outcome in terms of objective response and time to tumor progression. The FMCA significantly predicted objective tumor response with a sensitivity of 89% and a specificity of 53%. Furthermore, in patients with locally advanced breast cancer, low drug resistance was significantly associated with longer time to progression. It was concluded by the authors that the FMCA seems to report clinically relevant cytotoxic drug sensitivity data in breast cancer (Villman et al., 2005). Studies which examine the potential role of such testing are very helpful as physicians like those at Yale Cancer Center Melanoma Unit consider cost effectiveness, accuracy patient improvement statistics with their patient population.

If the medical practice of the Yale Cancer Center Melanoma Unit has evolved in a manner that the decision methods it uses to treat patients result in treatments similar to

those that would be indicated by an assay directed therapy, this would seem to indicate that heuristic rules may in fact arrive at similar results as those based on the standards advocated by those who favor evidentiary based medicine.

More generally, the medical profession has long been characterized by a learning curve. Novice practitioners are overly thorough in their patient histories, physical examinations, and use of diagnostic tests. As they progress in experience, they began to exhibit more directed use of each of these elements of forming accurate diagnoses as they become experts and practice at more of an expert level (Greenalgh, 2002).

Over the last century, as more advanced therapies accompanied by randomized trials of their effectiveness became available, the question of what it has meant to be practicing at an expert level has become a topic of much discussion in the medical profession. Some argue that experts have developed useful mental shortcuts that incorporate both the best clinical practice as well as diagnostic features in front of them (Sackett and Rennie, 1992; Andre et al., 2003; Gabby and le May, 2004; McDonald, 2005; Devereuax et al., 2005). Without this mental simplification, they would be overwhelmed by the complexity of practice particularly in situations requiring many complex decisions (Croskerry, 2002).

Others have reasoned that with the more rapid advancement of science, keeping practices and therapies up to date requires the ongoing incorporation of evidence based medicine into every day practice where possible (Sackett and Rennie, 1992; Devereaux et al., 2005). The relevant issue is that when rules of thumb are used in practice and yield good outcomes, they can be seen as a sign of expertise; however, when they lead to a

clinical mistake, they may be emblematic of bravado or a reliance on outdated training that needs the constant reincorporation of new knowledge (Croskerry, 2002).

Naturally, if evidentiary medical evidence is to be incorporated into a practice, it requires both the acquisition of knowledge and its appropriate use. Studies have been undertaken of the degree to which physicians in clinical practice avail themselves of the most recent evidence (Urquhart and Hepworth, 1996; Gabbay and le May, 2004) as well as whether they know how to interpret research results stated in the language of statistical theory (Steurer et al., 2002).

The results of that research generally indicate clinical physicians do make reasonably regular use of formal methods of acquiring knowledge while also relying on social networks within clinical or diagnostically related areas (Urquhart and Hepworth, 1996; Gabbay and le May, 2004). This suggests that understanding those social networks and training those most important in the actual pathways of information for clinicians is important in disseminating new knowledge into clinical practice. Nonetheless, there is also evidence that individual physicians seek diagnostic information on their own.

Unfortunately, research also shows that clinicians have difficult interpreting test results that are stated using common statistical terms employed in reporting results of clinical trials (Steurer et al., 2002). This casts doubt on the arguments of those who have argued that clinicians can operate in a manner such that they can take Bayes' Theorem, use local prevalence rates along with sensitivity and specificity in order to calculate a locally valid PPV of a test they are considering (Ashby and Smith, 2000).

The Yale Cancer Center Melanoma Unit does not undergo deficiencies in clinical practice that strict advocacy to only evidence based medicine would help overcome.

Members of the group are actively involved in research, participate in clinical trials, and have regular clinical interaction with each other and external experts. Both these individual efforts as well as organized group efforts continually update the practice to be at the frontier of the field.

Nonetheless, the group has developed an expert practice in an area that requires immediate treatment decisions in a complex setting. Thus, the literature regarding heuristic decisions becomes germane to the research presented here. This chapter offers a brief review of medical heuristics and the incorporation of evidence into practice.

Medical Heuristics in Clinical Practice

Elements of uncertainty are wound into every clinical case in medicine. Despite the abundance of technology, clinicians still must use their judgment when making clinical decisions. The role of judgment and the clinician's expertise relative to the use of formal tests or evidence from research or clinical trials embodies the tension between what has been referred to as heuristic decision-making and evidence based medicine. As stated by Clement McDonald MD, "The trick lies in knowing when to be parsimonious with the use of diagnostic testing and which situations demand a no-holds-barred pursuit of an answer."

While this decision ultimately is dependent on each individual physician, their knowledge, and the situation they face, many feel that if the rules of thumb clinicians use can be acknowledged, they can be examined with respect to outcomes just as other components of practice can be. McDonald discusses at length many of the common rules both physicians use daily, the evidence that generate them, and how the two are inconsistent.

For example, McDonald (1996) state that, of course, "An unassailable position is to treat abnormalities only when they are above the threshold at which clinical trials have shown benefit of treatment." However, although the benefits of many drug therapies have been established in randomized trials, precise information such as exactly when the therapy should be initiated and how long it should continue is not present. Moreover, how the therapy should be varied in the presence of the complexities of individual cases is seldom made clear.

McDonald describes the tension between clinician judgment in individual cases and treating relative to statistical standards calculated from a population. He describes well known clinical adages such as: don't treat the numbers and operate now to avoid greater risk in the future. McDonald argues that, "historical trends or statistical realties suggest either doing the opposite or investing in more discriminating heuristics." The author argues that rules of thumb used in practice need more refinement that would result in less practice variation and more efficient medical care (McDonald, 1996).

The primary concern about rules of thumb that some clinicians come to use in practice is that they may contradict known scientific evidence. The reason they do this can vary. They can be handed down during clinical training (Gabbay and le May, 2004) that has become dated, they may reflect a lack of time to spend accessing journals or an inability to correctly understand the information contained there (Urquhart and Hepworth, 1996), or other issues such as complacency and lack of professionalism. In contrast, Sackett et al. (1996) states that, "Evidence-based medicine is the conscientious, explicit and judicious use of current best evidence in making decisions about the care of individual patients."

Beyond these concerns and the arguments in favor of the incorporation of evidence based practices into medicine where possible (Sackett and Rennie, 1992; Devereuax, 2005), rules of thumb or heuristics can also be considered as empirical evidence of a link between science and practice in clinical medicine. It has been argued that there is an art involved in clinical practice which requires an interrelationship between biomedical and humanistic perspectives (Andre et al., 2002). According to this argument, incorporating those two dimensions defines a good clinician.

Andre et al. (2002) draw from work in cognitive psychology which has defined heuristics as "autonomic mental processes that are described as mental shortcuts".....and are considered useful and even necessary to guide search and choice in uncertainty and under time constraint" (Gigerenzer and Todd, 1999; Andre et al., 2003). The study itself consisted of ethnography of a geographically confined area in Sweden. They interviewed groups of physicians in order to elicit responses about rules of thumb they used in practice and how they felt they developed. They also asked about benefits and dangers of this practice approach.

The investigators found that the practitioners that they observed alternated between rules for somatic and psychosocial problems (Andre et al., 2003). These rules expressed not only the likelihood for disorder but also the assessment of risk. Some rules aimed to minimize the risk of missing a serious disorder as wide criteria for inclusion were used. On the other hand, some rules expressed the aim to secure the identification of the really sick patients with the calculated risk of failure to include every single test criteria. Their assessment of risk influenced the relative preference given to the generalizing and individualizing process. When the practitioner considered the risk of a

serious somatic complaint to be high, they tended to individualize the consultation less. The more emergent the clinical decision to be made, the less weight were the rules used to individualize the consultation. When the practitioner judged the risk of a potentially disabling disorder to be low, they gave more room for the individualizing process.

When practitioners self-describe their practice decision-making assessment, they consistently describe the use of clinical dichotomies as a starting point in the diagnostic process, e.g. urgent-non urgent or physical pathology or no physical pathology (McWinney, 1997; Andre et al., 2003) and Morrell writes that the first stage of the diagnostic process is to identify serious life-threatening disease (Morrell, 1991; Andre et al., 2003). Not every clinical situation is a quandary. The practitioners in this study described how they did not abandon either the somatic or psychosocial focus (Andre et al., 2003) which is the case as was observed in this evaluative study of the physicians evaluated in this study who treated malignant melanoma.

With psychosocial problems, the patient becomes the expert from whom the practitioner must learn more, whereas in somatic problems, the practitioner is the expert. In many cases of therapeutic decision, the clinician and the patient participate in the decision making. Together both consider the outcome probabilities and patient preferences. This form of clinical decision-making is best used for problems involving medical uncertainty (Frosch and Kaplan, 1999). The clinician-patient dyad considers treatment options and consequences and explores the benefits and consequences of treatment for various outcomes. Previous research (Frosch and Kaplan, 1999) had shown that patients did not wish to be involved in decision making, yet these studies typically failed to separate decisions about technical aspects of treatment from preferences for

outcomes. Previous research has identified the complexity of patient's needs in the modern era including a patient's desire to be part of therapeutic decision making and the priority that their clinician to be in sync and concerned with those needs and desires. While older studies make no mention of issues of patient autonomy (Fletcher et al., 1983; Schattner et al., 2004), a recent systematic review of the literature on patients' priorities regarding their clinicians found "humaneness" to be the most highly desired aspect of care, followed by clinical competence and patient's participation in decisions.

Schattner et al. (2004) found that most patients want to be informed about their health and to be involved with their care plans (Schattner and Tal, 2002; Coulter, 2003; Schattner et al, 2004). To do that, patients must have clear information, which takes into account their unique circumstances. Patients expect their clinicians to heed these needs and prefer clinicians who are sensitive to the varied aspects of patient's autonomy and patient's rights. Schattner et al. (2004) focused on selections of a population of 445 randomly selected in-patient and outpatients from a heterogenous population of 250,000 people regarding their preferences for different attributes of their physicians. The investigators used questionnaires with statistical results based on chi-square tests and one-way ANOVA or t-tests and found that while heterogeneous preferences existed, attributes in the domain of patient autonomy, physician's expertise, and physician humanness and support were chosen most. The studies of Thom et al. (2001) have already indicated that certain physician behaviors were important for patient trust. Patient trust was significantly correlated with compliance and with clinical improvement (Thom et al., 2002).

By and large, the diagnostic decision-making process is left to the clinician and in many circumstances consideration is given to the patient's contribution towards that end. When rules of thumb are used, problem solving is characterized by a stepwise simplification and narrowing of the problem. This is in contrast to the classical description of problem solving in consultation (the hypothetico-deductive method), which assumes a scientific method, where the clinician creates a hypothesis from patient cues and tests this hypothesis (Pendleton et al., 1984; McWinney, 1997; Croskerry, 2002; Andre et al, 2003). Further research has questioned this description and, instead, knowledge-driven problem solving has been revealed, where the expert in their clinical reasoning uses schemes specific to the problems in their domains. The availability and order of knowledge determine the usefulness of the expert reasoning (Mandin et al., 1997; Andre et al, 2003). The rapid assessment of emergency and psychosocial problems as well as separate rules for somatic and psychosocial problems could be part of the tailored scheme and expert knowledge used by the experienced clinician. In contrast to a deductive strategy, where the singular event is predicted form general laws, this is an inductive process, reasoning from a singular event.

Even advocates of greater adherence to evidence based medicine acknowledge that scientific data cannot be expected to guide most medical decisions. There are not nearly enough randomized trials or epidemiologic studies to guide all clinical decisions (McDonald, 1996). Not only are there not enough studies to address all etiology and diagnostics of clinical nuance, the job of tailoring such information to each patient renders the vast quantity of scientific data overwhelming to say the least. Extrapolation is used often in clinical practice, yet not consistently (ibid). It seems the current position

of the Food and Drug Administration (ibid) that a more consistent heuristic would uniformly adopt a stance on individual drugs based solely on the results of clinical trials. However, it does not take into account cost and the lack of effectiveness and accuracy of test results for every given situation and treatment. A better rule may be to allow extrapolation of therapeutic benefits from one drug to another if they are of the same physiologic class but do not across such classes. Bayes Theorem mentioned in an earlier discussion in this paper provides rationale for exactly this approach and will be further described here. The practice of medicine can achieve a blend of both the use of rules of thumb and quantitative analysis, such as Bayes Theorem.

Bayes' Theorem in Clinical Practice

The ongoing incorporation of new information into a medical practice requires that the results of research and clinical trials be assessed and that the relevant information be incorporated into the practice. As discussed in Chapter 2, the results of most clinical scientific research refer to the sensitivity and specificity of the research results. If a clinician knows the prevalence rate of the disease, Bayes' Theorem can be used to calculate the probability that a positive test result is indicative of the presence of the disease. This information can be incorporated into the decision making process of devising the most effective treatment plan for individual patient.

There are additional uses of Bayes' Theorem. Ashby and Smith (2000) provide a number of hypothetical examples of how the Theorem can be applied at different points in the health care system (Ashby and Smith, 2000). These more advanced formulations involve the use of utility maximization and/or cost theory from economics in conjunction with the Theorem. While application of these ideas in a research setting is interesting

their application may not be useful in the clinical setting. It seems unrealistic to expect the clinician adopting this explicit thought process in making treatment decisions.

Obviously, the use of Bayes' Theorem to calculate PPV and NPV of tests, or the adoption of those same values from research articles and incorporation of them into their practice requires several things. First, physicians must look to journals for evidence. Second, they must be familiar with the concepts. Finally, they must be able to interpret the information. What does research say about this?

Availability of Information through Databases

Beyond the standard measures of test results that are contained in journals, it is worth mentioning that the value of evidence based medicine is seen as being great enough that organized efforts are underway to collect information on the behalf of physicians. For example, randomized trials are regarded as the gold standard for evaluation of new therapies (Ashby and Smith, 2000). The Cochrane Collaboration aims to collate systematically all randomized controlled therapies and new diagnostic tests through regularly updated systematic reviews to provide the best current evidence on different therapies.

The pharmaceutical industry also contributes to the available base of knowledge for the practice of evidence-based medicine. In order to obtain marketing authorization for a product, pharmaceutical companies have to provide regulatory authorities with structured evidence to demonstrate the safety, quality and efficacy of the product, justifying the indications, the appropriate doses and any contraindications. While there are shortcomings of trials; such as the lack of evidence of long-term toxicity since most

trials are of a short duration, the drug approval process does result in useful clinical information.

In the United Kingdom, the National Institute for Clinical Excellence has been set up to appraise the relative merits of differing therapies (Department of Health, 1997; Ashby and Smith, 2000). This will be based on the available evidence, as are current licensing decisions, but also on cost-utility estimates (Freemantle and Mason, 1999; Ashby and Smith, 2000). This is already provoking vigorous comment (Grace, 1999; Ashby and Smith, 2000) and justifiably so. This latest direction of national health care involves values, utilities as well as clinical safety and efficacy. The explicit aim is the "involvement of patients and the public in decision-making."

Accessing Information: Social Networks

Although clinicians appear to make use of libraries in updating their practice styles, at least two studies also show that they rely on professional networks for information (Andre et al., 2002; Gabbay and le May, 2004). Both studies used ethnographies of groups of physicians to investigate whether they in fact used heuristic decision making processes. As part of their studies, they observed physician practices and would question how they arrived at some decisions.

One observation, perhaps best stated in the study of Gabbay and le May (2004) was that, "We found that the individual practitioners did not go through the steps that are traditionally associated with the linear-rational model of evidence-based health care – not once in the whole time we were observing them. Neither while we observed them did they read the many clinical guidelines available to them in paper form or electronically." In practices such as these, the question then arises of whether they take another route in

incorporating information into their practices. The answer was that the physicians updated their practice styles, "via their professional networks among other doctors." In one particular practice, "the local primary care trust pharmaceutical adviser had . . . earned the respect of the practitioners and was a highly trusted source."

Appropriate Use of Research Evidence

Clinicians appear to make use of multiple sources of information in updating their practices. To the extent they rely on published sources, this will require that they assemble, manipulate, and interpret the information correctly. Effective therapeutic action rests on the correct performance of each of these tasks.

A reasonable question is what evidence exists that clinicians can perform this task? Steurer et al. (2002) performed a randomized trial on this topic. The investigators set out to assess the extent to which different forms of summarizing diagnostic test information influenced general practitioners' ability to estimate disease probabilities (Stuerer et al., 2002). The investigators provided questionnaires to 263 general practitioners in Switzerland in practice on average for 10 years. The questionnaires contained multiple choice questions about terms of test accuracy and gave a clinical vignette with the results of a diagnostic test described in three different ways (test result only, test result plus test sensitivity and specificity, test result plus the positive likelihood ratio presented in plain language). They find that in instances where the prevalence of disease is low, most doctors grossly overestimate the probability of disease in patients with a positive result from a screening test, charging that the doctor confuses the sensitivity of a test with its positive predictive value. Despite a long tradition of reporting test accuracy in terms of sensitivity and specificity, only a minority of the participants in

this study could correctly apply the information. They conclude that the difficulty in performing the required calculations probably explains their lack of use in general practice (Reid, Lane and Feinstein, 1998; Steurer et al., 2002).

There has been a long tradition of reporting test accuracy in terms of sensitivity and sensitivity. This Swiss study raises the question as to what extent overestimation of the diagnostic value of screening procedures contributes to the steadily increasing use of laboratory and imaging tests. Studies that investigate this question may well be lacking in their design and while the Swiss study involves questionnaires for only a small test sample, it raises an important issue in the move towards evidence based medicine.

Diagnostic Judgment

While it is important to carry the appropriate knowledge from clinical evidence, drug trials, and medical evidence into everyday practice, a patient's unique predicament warrants careful consideration of the facts specific to their individual circumstance and need. Each clinical encounter renders itself unique and of all of the diagnoses that ever will be made, most are made during the history the rest during the physical examination. For example, Crombie (1963) documented that 88% of diagnoses in primary care were made by the end of the completion of the patient history and physical exam (Crombie, 1963; Sackett, 1992) Similarly, Sandler (Sandler, 1980; Sackett, 1992) found that 56% of patients in a general medical clinic had been assigned correct diagnosis by the end of their history, and that this figure rose to 73% by the end of their physical examination. Even when patients are referred to specialists, additional history taking is performed along with physical examination (Sackett and Rennie, 1992).

While there are no doubt clinical encounters where a physician might resort to reference materials in making a diagnosis, often, the initial examination is sufficient to establish a diagnosis. The examination provides the necessary data, and as additional information is acquired the clinician moves down diagnostic pathways arriving at a diagnosis.

Revisiting Therapeutic Decision Making

Similar processes are conducted when a clinician makes therapeutic decision about drug treatment choice. Patient substrate along with clarity of presentation has significant impact on a clinician's willingness to accept clinical uncertainty. Therapies that carry significant risk are reserved for more serious illnesses such as cancer. They are administered when there is some reasonable certainty that the patient is suffering from the disease or are doing so poorly that it is felt to be worth the risk. Treatments that carry less risk are initiated with relative impunity and too can have their downsides. In the case of malignant melanoma, patients likely have significant baseline organ dysfunction and often have little reserve and therefore tolerate additional insults poorly.

This imparts a sense of urgency to the process which is generally absent when caring for patients with less serious medical concerns or conditions. While tests are being obtained to define the nature and extent of a clinical problem or aid to making treatment determination, empiric therapies are initiated in an effort to prevent further deterioration. If no tests are available that can accurately aid in therapeutic decision, a number of therapeutic strategies may be pursued in parallel or therapy is administered based on clinical experience and their understanding of existing clinical research evidence.

Patients in the advanced stages of melanoma who are administered chemotherapy and immunotherapy often require very close monitoring so that appropriate adjustments can be made and clinical turn-downs rapidly addressed. This mentality is less appealing and elegant then focused therapy and "solid" clinical evidence, but must be given specific attention where interventions are initiated individually, enabling the clinician to clearly gauge their efficacy and minimize associated therapeutic risk.

Clinical decision making is based on the expectation that the human body will respond to an illness in a predictable way. Disease states which alter this behavior wreck havoc on logical decision making. Illnesses which decrease normal immune function, medications that blunt the response to infection, or advanced age are a few examples. When caring for these patients, clinicians are forced to cast a wide net, relying on the initiation of empirical therapies while multiple diagnostic and therapeutic avenues are pursued. There will always be some element of non-modifiable uncertainty in clinical medicine. Technological advances have succeeded in improving both the diagnostic and therapeutic accuracy. However they are almost never correct all of the time. Reliance on the less-than-perfect information that tests provide without taking into account clinical judgment can have serious consequences to the patient.

One parameter that is a constant in the mind of the clinician is quality of life. The decision making process for the clinician includes the effects of diagnostic testing and the therapeutic decision will affect the patient and their circumstances. Quality of life is a rather large and encompassing category that incorporates emotional well-being of the patient and family, work, social, economics, the immediate and future impact of decisions, personal preferences and may include religious or faith practices. Each

individual patient represents a different set of needs under this category. At the time of therapeutic decision making, the clinician is also called to impart these specifics into the decision making process. This can be a straightforward task or can become more involving, if for example there is financial hardship or a patient may require in-patient hospitalization for drug administration yet feels obligated to tend to family needs. The varying scenarios are numerous and not easy to measure or quantify (Guyatt, Bombardier and Tugwell, 1986a), yet they become part of the equation when examining and evaluating decision making with respect to the clinician and the patient.

Conclusion to Chapter 4

The literature regarding the issue of heuristics and evidence based medicine has an adversarial quality to it which leads one to believe that one side wants to win at the expense of the other, that this is a zero sum game. However, the clinical practice of medicine is not an all-or-none process where rules of thumb and clinical evidence operate in exclusive competing domains.

The need for follow-up to date knowledge is irrefutable in order to provide the best care to patients; however, there is an art involved in translating that knowledge in a usable form into clinical practice. Traditionally, a novice clinician would rely too much on a detailed history, overly rigorous examine, and excessive utilization of diagnostic tests. As the expertise of a clinician rose, the utilization of their experience and personal clinical judgment allowed them to understand which elements of these practices were appropriate in each context – a balance was sought between knowledge the physician might seek and their own sense of what was appropriate in context.

Heuristic practice styles relative to the use of evidentiary medicine are simply drawing out threads that have always been interwoven in a quality, professional practice. Judgment must be exercised in accessing relevant knowledge for each situation and sound clinical decisions rely upon correct information. Over-reliance on either element or the suggestion that one should come at the expense of the other seem out of place.

In the context of a diagnosis of stage III or stage IV malignant melanoma, this observation seems especially true. Clinicians at the front line of treating the disease are clinically involved in achieving more effective therapies and keeping abreast of medical literature. The prognosis of the diagnosis brings the sobering reality of mortality in constant confrontation. Interface with patients and the reality of their lives in this context requires a balance of heuristics and evidence-based medicine as observed in the Yale Cancer Center Melanoma Unit. The findings from the evaluation of the group and its unique style may serve as a model for future studies for design models for treatment decision making for melanoma or other cancers.

The final chapter will summarize the current state of therapeutics for malignant melanoma and including discussion of promising treatment utilizing lymphodepletion and autologous T-cell transfer regimens. The discussion includes approaches to evaluating new therapies in melanoma and the future role of EDR assays. Issues pertaining to social and economic responsibilities of companies within the context of a global approach to utilization of diagnostic testing and drug prescription are also discussed. The concluding section will propose a model for a melanoma unit using the elements and data as defined in the study.

CHAPTER 5: IMPROVED APPROACHES TO MELANOMA THERAPIES

The rapid increase in incidence of malignant melanoma over the last decade has not been accompanied by an increase in improved therapeutic options. Single-agent chemotherapy or immunotherapy remains the treatment of choice when systemic therapy is warranted. Dacarbazine (DTIC) is the chemotherapy of choice with a response rate of 16% (Atallah and Flaherty, 2005). Other chemotherapies including cisplatin, paclitaxel, docetaxel and the DTIC analog temozolomide, have shown activity in this disease (Atallah and Flaherty, 2005). Based on their single-agent therapy, several combination chemotherapies have been investigated with preliminary results that appeared promising. However, in randomized phase III trails the two most active chemotherapy combination regimens, cisplatin, vinblastine, and DTIC (CVD) and the Dartmouth regimen (DTIC, cisplatin, bischlorethylnitrosourea, and tamoxifen), did not display superiorority to single-agent DTIC for overall survival (Atallah and Flaherty, 2005). Immunotherapy with either interleukin (IL)-2 or interferon (IFN) has demonstrated response rates or 10-15% in appropriately selected patients. In patients who achieve a complete response, responses can be of greater durability than those with chemotherapy. However, IL-2 and IFN administration are associated with multiple side effects, and only physicians and clinicians experienced with management of such therapies should administer them. The potential benefit of combining chemotherapy with immunotherapy has led to multiple phase II trials of biochemotherapy that appeared to be associated with higher response rates and longer median survivals (Atallah and Flaherty, 2005).

Several phase III trials have been completed that have not consistently demonstrated an improvement in either response rates or overall survival, and these

approaches to therapy cannot be routinely recommended outside of the context of a clinical trial. The surgical resection of isolated metastatic disease has demonstrated an important palliative benefit to those patients who present with solitary and symptomatic bony metastasis. Both sterotactic radiosurgery and whole brain radiotherapy have been used alone and in combination to benefit patients in this troubling clinical circumstance. Isolated limb perfusion and a newer technique, isolated limb infusion have demonstrated high response rates for those uncommon patients who develop recurrent disease isolated to a limb. According to Atallah and Flaherty (2005), if complete metastasectomy is not feasible and in the absence of brain metastasis, single agent IL-2 is a good initial treatment choice in appropriately selected patients. Single-agent chemotherapy with DTIC is the treatment of choice for patients who are not candidates for IL-2. Adoptive immunotherapy combining nonmyeloablative chemotherapy with high-dose IL-2 is a potentially promising therapeutic strategy under investigation. Targeted therapy is also an area of promising development as single agents, in combination, and combined with chemotherapy.

The goal of treatment for metastatic melanoma remains one of palliation with a minimum of unwanted side effects and disruption to quality of life. The achievement of durable responses with biological agents and the possibility to complement the higher response rate of chemotherapy by prolonged duration of responses led to developments with biochemotherapy. In the adjuvant setting, immunotherapy has been shown to have important effects in relapse-free (Penhamberger et al., 1998; Grobb et al., 1998; Kirkwood et al., 2000; Hakansson et al., 2003) and overall survival (Kirkwood et al.,

1996; Kirkwood et al., 2001; Hakansson et al., 2003) with a subset of durable and valuable remissions to suggest a future role in therapeutics.

The success of these therapies yields more information about generalized mechanisms of antitumor host response. Although a clear response rate (40-60% OR) has resulted in some studies of the combined modality, several phase III studies have mixed results on the duration of survival and various timeframes between the administration of chemotherapy and biologics have been tested, ranging between concurrent biochemotherapy, one day and up to three weeks (Alexandrescu at al., 2005). An analysis of the trend of responses and survival versus the duration of chemobiotherapy sequencing shows that, as the timeframe between chemo and bio components increases, the OR (overall survival), survival of (CR) complete responders and survival of (PR) partial responders appear to increase, but the effect is only present for the chemo-bio, and not for the bio-chemo sequence. Because there is no current explanation for this observation, it appears possible that the interaction between components of biochemotherapy results in a double effect: an increase in the immediate response reflected in the OR, CR, PR on one side, and an increase in survival on the other side (Alexandrescu at al., 2005).

Much progress has been made in the understanding of the properties of T cell subpopulations associated with states of T cell differentiation in mice and humans, especially those states that are related to the generation of memory T cells capable of protecting against viral challenge. However, little progress has been made in identifying the characteristics of cell states that are associated with the successful treatment of large, established tumors in mice or in humans (Appay et al., 2004; Masopust et al., 2004; Maus

et al., 2004; Sallusto et al., 2004; Gattinoni et al., 2005). T cell differentiation is a progressive process characterized by phenotypic and functional changes. Recent studies suggest that memory T-cell differentiation continues for weeks or months following antigen clearance (Masopust et al., 2004) although commitment to the memory lineage seems to occur at the effector stage of development. Several variables associated with priming, such as the duration of antigenic stimulation, degree of co-stimulation, cytokine environment, and CD4+ T cell help, may program epidemic qualitative differences into the ensuing effector and memory populations (Masopust et al., 2004). Defining what memory qualities best protect the organism from re-infection, as well as how commitment to the memory lineage is specified following T-cell activation remains an important goal. Research that examines the possible role of adoptive transfer therapy for the treatment of malignant melanoma is being presently conducted as discussed in the next section.

Adoptive Transfer in Malignant Melanoma Therapy

A recent therapy utilizing in vitro testing for malignancies including melanoma is called adoptive transfer therapy (ACT). It involves the administration of in vitro-activated and –expanded autologous tumor-reactive T cells, is currently one of the few immunotherapies that can induce objective clinical responses in significant numbers of patients with metastatic solid tumors (Childs and Barrett, 2004; Dudley and Rosenberg, 2003; Rosenberg et al., 2004; Gattinoni et al, 2005). Dramatic clinical responses were observed following the adoptive transfer of autologous tumor-infiltrating lymphocytes (TIL) in a patient studied by Robbins et al. (2002). Clinical investigators are devising strategies to enhance the development of adoptive transfer of activated effector cells or

tumor-specific antibodies into the tumor bearing host (passive tumor immunity). The identification of T cell-defined tumor antigens in melanoma has led to the development of clinical trials that target cancer cells by augmenting the antigen-specific cellular immune response Van den Eynde, 1997; Yee et al., 2002).

In a study previously conducted by Dudley and Rosenberg (2002), ACT lymphodepleting conditioning caused objective responses in 46% of patients with metastatic melanoma refractory to other therapeutic modalities. Autologous cell transfer after lymphodepleting chemotherapy can cause the regression of large, vascularized tumors in patients with refractory metastatic melanoma.

A more recent study by Rosenberg and Dudley (2004) was triple the size of their original study. The outcome objective rate exceeded 50% and 11% of all patients treated were complete responders. Eighteen of 35 patients treated with tumor-reactive lymphocyte cultures experienced an objective clinical response (>50% reduction in tumor), including four complete responders. In some patients, tumor regression was accompanied by large in vivo expansion of the administered antitumor lymphocytes, which persisted in peripheral blood at >70% of total lymphocytes for many months after transfer. The cells capable of mediating tumor regression consisted of heterogenous lymphocyte populations with high avidity for tumor antigens that were derived from TILs cultured for limited times in vitro. The success of this treatment likely result from the ability to infuse large numbers of activated antitumor lymphocytes into an appropriate host homeostatic environment depleted of regulatory T cells. These studies are elucidating the requirements for successful immunotherapy of patients with advanced

metastatic melanoma disease and are leading to additional clinical trials with gene-modified lymphocytes.

In another such study that combined nonmyeloablative lymphodepleting chemotherapy with adoptive transfer of tumor-specific CD4 and CD8 T cells, exhibited an initial objective response rate of 51% for patients with stage IV melanoma (Poehlein et al., 2005). While this newer therapy strategy is extremely challenging, future studies may be uncovered that simplify these approaches and further improve outcome.

There are several theoretical advantages to the use of ACT in the treatment of cancer, including malignant melanoma. The tumor-specific T cells can be activated and expanded to large numbers in vitro, independently of the immunogenic properties the tumor. In this way, in vitro drug testing can be enhanced and continue to find a potential role in advancing therapeutic improvement. Perhaps the most important benefit to ACT is that the functional and phenotypic qualities of T cells can be selected prior to their adoptive transfer.

Currently the only criterion applied to selecting cells for adoptive transfer to patients with solid tumors is the ability of antitumor T cells to release INF-α and kill tumor cells upon co-culture (Dudley and Rosenberg, 2003; Gattinoni, 2005). However, based on a retrospective analysis of ACT in melanoma patients, it is now clear that these criteria alone do not predict in vivo efficacy-despite enhanced in vitro antigen-specific IFN-α release and cytolysis, tumor-specific CD8+ clones did not induce objective clinical responses upon adoptive transfer. In this regard, in vitro testing does not bode as well for ACT.

Gattinoni et al. (2005) evaluated the efficacy for adoptive immunotherapy by transferring tumor-specific CD8+ T cells into tumor-bearing mice at various stages of differentiation. Gattinoni et al. found that administration of naïve and early effector T cells, in combination with active immunization and IL-2, resulted in the eradication of large, established tumors. Despite enhanced in vitro antitumor properties, more-differentiated effector T cells were less effective for in vivo treatment. Several events may underlie the paradoxical phenomenon: (a) down regulation of lymphoid-homing and costimulatory molecules; (b) inability to produce Il-2 and access homeostastic cytokines; and (c) entry into a proapoptotic and replicative senescent state. While the progressive acquisition of terminal effector properties is characterized by pronounced in vitro tumor killing, in vivo T cell activation, proliferation, and survival are progressively impaired. These findings suggest that the current methodology for selecting T cells for transfer is inadequate and provide new criteria for the generation and screening of optimal lymphocyte populations for adoptive immunotherapy (Gattinoni et al., 2005).

In favor of the in vitro testing in this method, it seems that Gattinoni et al. (2005) were able to confirm in their study that impaired cells do differentially express genes that are associated with highly differentiated effector cells. The expression of many genes involved in effector functions in the study, were strongly upregulated in impaired cells compared with in vivo effective cells. However, highly differentiated T cells overexpressed a number of genes that may prejudice their survival capability, such as proapoptotic molecules. Intermediate effector cells in the study, expressed higher levels of molecules associated with replicative senescence, suggesting that the proliferative potential of these cells may be decreased. The micro array data in the study were

validated by flow cytometry, Western blot, and functional analysis. The Western blot was found to confirm and further refine the results of the analysis of global gene expression.

T cell differentiation is associated with progressive impairment of proliferative capacity. The analysis in the study by Gattinoni et al. (2005) of gene expression suggested that fully differentiated effector T cells may be less "fit" and more proapoptotic. In vitro testing was performed using CFSE-based assay to determine the relative proliferative capacity of effector T cell subpopulations. To test whether the in vitro findings could predict the proliferative capacity of T effector subsets upon adoptive transfer, the absolute numbers were directly quantified (CD8+specific T cells) at multiple time points in the blood and in the spleen. The investigators found that the differences observed in vivo were more pronounced than those observed in vitro. This finding suggests that the progressive acquisition of antitumor effector function led to deterioration of the numbers of surviving T cells in vivo.

T cells used for current adoptive immunotherapy trials are selected for their capacity to produce high levels of IFN-α and for their ability to efficiently and specifically lyse relevant target cells (Dudley and Rosenberg, 2003; Gattinoni et al., 2005). According to Gattinoni et al, CD8+ T cells that acquire complete effector properties and exhibit increased antitumor reactivity in vitro are less effective at triggering tumor regressions and cures in vivo. As discussed previously, findings such as those in this study indicate that emerging data from human clinical trials (Gattinoni et al., 2005) support the hypothesis that less-differentiated T cells are more therapeutically effective upon adoptive transfer.

After adoptive transfer, several events must occur for T cells to cause the regression of established tumors (Gattinoni et al., 2005). (a) T cells must be activated in vivo through antigen-specific vaccination. (b) They must then vigorously expand to levels capable of causing the destruction of significant tumor burdens. (c) Antitumor T cells must survive long enough to complete the eradication of all tumor cells.

Homeostatic cytokines are important for the survival of T cells (Gattinoni et al., 2005). For example, IL-7 has been shown to be critical for the generation and survival of memory CD8+ T cells. The investigators found that the induction of apoptosis of activated effector Cd8+ T cells is required to maintain lymphoid homeostasis and to prevent the development of autoimmune manifestations and lymphoid neoplasia, but apoptosis after adoptive transfer is clearly undesirable.

The idea that highly differentiated T cells are suboptimal to confer immunity is also supported by recent data from HIV patients. The incidence of IFN-α+perforin ^{high} HIV-specific CD8+ T cells has been reported to correlate with HIV-1 disease progression (Heintel et al., 2002; Papagno et al. 2004; Gattinoni et al., 2005).

Gattinoni et al (2005) raise pertinent questions based on their study regarding the optimal strategy for the in vitro generation of T lymphocytes for adoptive transfer. The mere generation of large numbers of highly differentiated T cells for adoptive transfer is insufficient to trigger tumor regression. High doses of IL-2 have been used to obtain large numbers (up to 10^{11} cells) of tumor-reactive T cells for adoptive transfer, but T cells rapidly differentiate under this condition. In contrast, IL-15 uncouples differentiation from proliferation, enabling the generation of large numbers of less-differentiated/more effective T cells. These findings are now being applied to current clinical efforts in the

treatment of established tumors and may be useful in the treatment of established infectious diseases, including HIV and chronic hepatitis. Great progress is being made in the field of tumor immunology in the past decade, but optimism about the clinical application of currently available cancer vaccine approaches is based more on surrogate endpoints than on clinical tumor regression (Rosenberg et al., 2004).

Considerable advances have been made in the field of allogenic hematopoietic stem cell transplantation (Childs and Barrett, 2004). Recognition that transplanted donor immune cells can cure patients with leukemia has led to the development of nonmyeloablative or "low-intensity" conditioning regimens, which have expanded the application of allogeneic transplantation to a growing number of hematologic malignancies. The improved safety and preliminary success of this transplant approach have justified applying allogeneic immunotherapy to patients with treatment-refractory solid tumors.

Proliferation, neovascularization, lymphangiogensis, invasion, circulation, and embolism are important steps in the pathogenesis of melanoma metastasis. Tumor vascularity has proven an important independent significant prognostic factor. The first determinant of treatment is the histologic diagnosis. The process of determining the staging plays an important role and is the second factor in making therapeutic choices. The patient's probable tolerance for the side effects of the various possible treatments is the third factor. Finally, assessing the specific needs of the patient, their baseline health and their autonomy is inclusive to the treatment plan. A decision to treat with curative intent demands a higher degree of adherence to drug dosing and scheduling requirements and acceptance of treatment-related toxicity.

When cure is not a realistic expectation, a decision to treat is based on an expectation for prolongation of the patient's life or an improvement in the quality of life. In these cases, treatment-related side effects may be minimized by dosage adjustments or treatment delays when necessary, but at the cost of antitumor efficacy. When standard treatment is found to be ineffective or the diseases advances to stage IV melanoma, clinical trials are recommended. While quality of life can be difficult to measure accurately, investigators should no longer shy away from including it as an outcome in studies designed to determine treatment benefit (Guyatt, 1986a). In consideration of the individual treatment plan for those with advanced melanoma, palliative care may be the desired therapy for a fair portion of patients and quality of life issues may foreshadow the desire to increase survival duration.

The economic benefit to reducing ineffective chemotherapeutics provides rationale and offers validity to pursuit of diagnostic testing methods to augment treatment decision making in consideration of their utilization in the approach to disease management.

The evolution and progressive refinement of an internationally accepted melanoma staging system over the last 50 years has resulted in much greater accuracy and increased utility, but the process has become more complex and less intuitive. This raises the question of whether melanoma staging should continue to develop with ever-increasing levels of complexity, or whether attempts should be made to produce an alternative system that is simpler and more intuitive. The current TNM-based AJCC staging system for melanoma incorporates only some of the prognostic factors thought to be significant. There are other prognostic factors being studied and considered which

may allow for accurate prediction of an individual melanoma patient's prognosis as has been discussed in this paper.

Recent advances in the fields of tumor biology, immunology, and molecular biology have provided the basis for the development of strategies targeting cancer at the molecular level. Although many of these attempts were extremely successful at the preclinical stage, their translation to the clinic proved to be more difficult than originally expected.

Limitations in obtaining clinically relevant responses are, at least in part, imposed by the extreme drug resistance seen in malignant melanoma. The growing knowledge about the genetic basis of melanoma provides one avenue for continuing the development of improved therapeutic strategies as does the progress made in the field of identification of melanoma antigens.

Notorious for its proclivity for metastasis and resistance to known therapies, malignant melanoma represents a major health concern. Genetic, epidemiological and genomic investigations are highlighting a repertoire of stereotypical mutations that are associated with human melanoma genesis (Kabbarah and Chin, 2006). The functional significance of many of these genetic alterations is being ascertained through the use of in vivo mouse models. Insights from human and mouse models, coupled with development of novel tools for high-resolution characterization of the melanoma genome, hold promise for the identification of better diagnostic markers and potential therapeutic targets.

Antiangiogenesis Agents

Melanoma is a highly vascular tumor and as such should be a good target for antiangiogenic therapy. Interferon has antiangiogenic activity, but it remains unclear how it operates. Thalidomide by itself has limited activity in metastatic melanoma (Eisen et al., 2000; Lange et al., 2004). However, Hwu and colleagues (Hwu, 2000) combined it with temozolomide and obtained one complete response and four partial responses in 12 patients, including some with brain involvement. A follow-up study is presently underway (Balch et al., 1981; Lange et al., 2004). Thalidomide in combination with chemotherapy is well tolerated and as shown an increased clinical efficacy compared with chemotherapy alone (Danson and Lorigan, 2005). The pro-apoptotic agent oblimersen has also shown improved progression-free survival and response rate, although not overall survival, when combined with DTIC compared with DTIC alone (Danson and Lorigan, 2005). New antiangiogenic agents in melanoma trials include thalidomide analogs (Mariott et al. 2003; Lange et al., 2004), anti-vascular endothelial growth factors (Carson et al., 2003; Lange et al., 2004) and anti-alpha (v) beta3 (Natali et al., 1997; Lange et al., 2004) agents.

Cytokines

A number of cytokines in addition to IL-2 are being investigated in metastatic melanoma. These include IL-1, IL-4, IL-12, and IL18 (Triozzi et al., 1995; Lange et al., 2004). None has proven beneficial for patients with melanoma.

Vascular endothelial growth factor

(VEGF) is one of the new biochemical methods for noninvasive tumor therapy evaluation to determine whether the chemotherapeutics is effective (Cornelissen et al.

2005). Vascular endothelial growth factor is labeled with radioiodine and evaluated in vivo and in vitro using a melanoma cell line overexpressing VEGRF-1 and -2. Results from studies of athymic mice demonstrate in these internalization assays indicate that the preserved ligand induced internalization and metabolization of the tracer showing low background activity and a tumor to reference a positive tissue ratio. The results suggest that VEGF-1 may be used as a potential and suitable tracer for tumor therapy.

Vaccines

The idea that a vaccine can very specifically activate the immune system against melanoma without significant toxicity is very appealing to both clinicians and the patients. Identification of the most relevant tumor antigens will continue to be a vital component of vaccine design. While many vaccines are under preclinical and clinical evaluation, none has been proven to work in the adjuvant or metastatic setting in a randomized trial. It is thought that if vaccines are to prove effective, they will do so in the low-tumor-volume adjuvant setting or in the metastatic setting when combined with other therapies (Kadison and Morton, 2003; Lange et al., 2004).

It is thought that delivery of the antigens that are under current study can be optimized through use of adjuvants, dendritic cells, or heat shock proteins to enhance the immunogenicity of vaccines. The use of DNA vaccines to deliver nucleotides that encode relevant antigens and immunologic molecules and the use of targeted therapy with immunocytokines have yielded promising results in animal studies (Kadison and Morton, 2003). Several large randomized studies using allogeneic melanoma vaccines have shown minimal benefit and phase I/II studies with gene transfected melanoma cells do not seem particularly encouraging. Another observation regarding melanoma vaccines

is that the absence of severe side effects has the potential to provide a quality of life superior to standard multi-agent chemotherapy given the comparative side effects of the two approaches (Kuhn and Hanke, 1997).

It can be expected that the most effective antigens and method of administration will become apparent over the next few years (Hersey, 2002). Cutting-edge techniques such as quantitative polymerase chain reaction and gene/protein micro-arrays will be used to monitor the response to a vaccine which may offer guidance to treatment management decisions (Hersey, 2002; Sabel and Sondak, 2002; Kadison and Morton, 2003).

New Is Not Always Better

Predictive tests that can be shown to effectively identify patients with a high probability of responding may be useful in helping the physician determining the treatment regimen. As clinical responses are often only minor or partial and/or of short duration, there is also a need for better understanding of the mechanisms of action during treatment to be able to monitor the relevant anti-tumor activity during treatment and to optimize the efficacy of future possible therapeutic agents.

The availability of innovative testing is a constant in the clinical practice of medicine. The various considerations given to their efficacy and predictive value have been discussed in this paper with attention given to the physicians in the Yale Cancer Center Melanoma Group who treat melanoma.

This study evaluated decision making and associated variables involved in the clinical utilization of the testing methods in order to form conclusions regarding treatment determination. Effective health care services rely on a foundation of research-

based evidence. While quality of care improvements is dependent on the application of evidence, incorporating them into practice may be challenging (Guyatt et al., 1986a). The process of clinical decision making is multi-factorial and the burden placed on clinicians in incorporating all newly available tests into their practices has been discussed in this paper. "The continuing proliferation of medical technology renders the clinician's ability to assess articles about (diagnostic) tests ever more important." (Jaeschke et al., 1994)

The research presented here did not support the hypothesis that clinicians do not use the results of in vitro testing. Through the evaluation of utilization of Oncotech Inc. in vitro EDR testing as collated from medical records, physician interviews and participation of Weekly Melanoma Conference for this study, it was determined that overall the physicians did consider the results in this evaluative study.

There was evidence to suggest that the utilization of the results from Oncotech in the treatment of metastatic melanoma may affect cost to the patient, the physician and to society. Oncotech Inc. claims to avoid direct costs of ineffective therapies and costs of managing treatment related morbidity. The data in this study offers nominal evidence to support the claim from Oncotech Inc. yet this claim nor the hypothesis were not proven in the evaluative study. The suggestion that utilization of EDR testing in treatment determination for advanced melanoma will lead to cost reductions may offer incentive to conduct further evaluation.

There is no chemotherapeutic agent that has been proven to significantly increase survival rates in metastatic melanoma in recent literature or practice. This study did not disprove the hypothesis that utilization of Oncotech Inc. in vitro drug resistance test

results will increase survival. The results suggest that further evaluation of the utilization of in vitro diagnostic testing for drug resistance and biomarker identification for drug resistance may augment the determination of the therapeutic plan for the individual patient. Studies are underway that seek to uncover the mechanisms involved in the metastasis and tumor progression of melanoma cells. Molinari et al. (2005) investigated the possible relationship between P-gp and CD44 cell adhesion molecule involved in invasion as analyzed by the 'transwell chamber invasion" in vitro assay to demonstrate drug resistance. Further in vitro studies may offer discovery of mechanisms of drug resistance in melanoma cells which may then translate to improved therapies.

The study raises a relevant question in relation to evidence based medicine: Why are there some less solidly supported testing innovations widely adopted while others with apparently stronger support underused? Few studies have monitored change in preference practice over time to determine the sustainability of change. Research from other behavior change literature shows that initial change is difficult to maintain, with reported relapses as high as 80% (Cockburn, 2004). According to Ferlie et al. (2000), there is a weak relationship between the evidence base and its diffusion. The diffusion and take-up of scientific evidence has been shown to be both socially constructed and complex, a process which has been contested, where the identification of a tacit expert knowledge is the key power resource in shaping the way research evidence influences clinical practice.

According to Sackett the best evidence comes from the integration of individual clinical expertise with the best available external clinical evidence from systematic research (Sackett et al., 1996). By individual judgment he means the proficiency that is

acquired through clinical experience and clinical practice. This is accomplished especially through "more effective and efficient diagnosis and more thoughtful identification and compassionate use of the individual patients' predicaments, rights, and preferences in making clinical decisions about their care". By best available external clinical evidence he means clinically relevant research, often from the basic sciences, but especially from patient centered clinical research into the accuracy and precision of diagnostic tests, the power of prognostic markers, and the efficacy and safety of therapeutic regimens.

External clinical evidence can invalidate previously accepted diagnostic tests and treatments and replace them with new ones that are more powerful, more accurate, more efficacious, and safer. Good clinical practice incorporates both individual expertise and the best available external evidence, and neither alone is enough. According to Sackett, "without clinical expertise, practice risks becoming tyrannized by evidence, for even excellent external evidence may be inapplicable to or inappropriate for an individual patient. Without current best evidence, practice risks becoming rapidly out of date, to the detriment of patients."

Patient's preferences remain integral to modern evidence-based practice (Haynes, Devereaux and Guyatt, 2002). Schattner et al. (2004) found that while heterogeneous preferences existed, attributes in the domain of patient autonomy, physician's expertise, and the humane nature of the physician and support were chosen most.

To recap what Jaeschke said about the validity and utilization of diagnostic tests under consideration for utilization by the oncologist and surgeon who treat malignant melanoma, the ultimate criterion for the usefulness of such a test is whether it adds

information beyond that otherwise available, and whether this information leads to a change in management that is ultimately beneficial to the patient (Guyatt et al., 1986). Cancer treatment is a complex process and the treatment determination depends on a correct diagnosis and evaluation of several factors that can influence outcome and each case must be evaluated on an individual basis.

Timar and colleagues (2005) have introduced a cost-effective in vitro assay to determine drug resistance of pediatric leukemias, the findings of which are published from the National Oncology R & D Consortium in 2004. The investigators are using global genomic approaches the gene signature of malignant melanoma showing that Ca-channel blockers and EGFR tyrosine kinase inhibitors are effective in preclinical human melanoma models in breaking the apoptosis resistance of this tumor. In vitro drug-response testing appears to offer an additional and reliable piece of information about drug resistance status of a patient's tumor and is well worth the consideration of further study.

Social and Economic Concerns in Diagnostic Test Utilization

Novel diagnostic tests should include social and economic responsibility built in to the development of the product including its structure for use and availability of use by clinicians in the general population on global scale. The need to discover and develop diagnostic tools to improve upon timely detection of tumor resistance and biomarker identification of individual tumor behavior in the laboratory setting spans across the geographic distribution of the globe. Malignant melanoma is of concern in nearly every country in the world.

Issues of implementation and analysis include reaching the target population and developing design approaches to test the intervention are important variables for any company involved in product design for clinical use, especially if it will potentially be utilized in developing countries. In order for such tests to enter into the fabric of developing countries the diagnostic tests and the companies that make them must be reputable enough socially and economically to cross country lines. For example, screening for HIV in South Africa has been next to impossible as policy makers there have consistently created the biggest obstacle to implementation of appropriate prevention and therapeutic programs (Hasnain, 2004). Only recently, people within the government and ruling party, denying previous policy have agreed that antiretroviral drugs should be given to pregnant women with HIV. The social fabric of South Africa is markedly different from that of Western countries. Such cultural challenge is a reality and to truly become a strong company that intends to permeate into the global climate demands cultural sensitivity and adhering to the norms of the country or bringing a solid platform of intended product to the medical arena within that country.

Cost and economic burden are of universal concern in heath care. One benefit to conducting evaluations to compare and contrast similar diagnostic tests is to determine cost-effectiveness and feasibility in routine laboratories in developing countries. Both antimicrobial susceptibility testing and chemotherapy and immunotherapy testing strive to provide guidance for appropriate treatment. There is a need for simple, reliable and cost-effective methods for susceptibility and resistance testing in general. The data in the study in this document may offer information for future studies where comparisons may be made to compare and contrast EDR in vitro testing of malignant melanoma with

regard to cost once alternate like tests are developed. For example, recently Bala et al. (2005) compared the results of two methods of susceptibility testing, minimum inhibitory concentration (MIC) values by E test with disc diffusion results by the Australian Gonococcal Surveillance Programme (AGSP) method in Neisseria gonorrhoeae isolates. Both methods were easy to perform and gave reproducible rates. However, disc diffusion was cost-effective and more feasible in routine laboratories in developing countries like India.

In order to maximize the benefits of predictive testing, potential ethical and social risks must be identified and minimized. Necessary steps include providing adequate information for patients and families, preparing them to receive test results; maintaining confidentiality' preserving the principal of solidarity to provide assurances of medical care and social support (Mehlman, 2004). Treating physicians and clinicians involved in genetic testing as it likely will accompany biomarker testing and in vitro testing for malignant melanoma must play a significant role in helping individuals, families and society in general to make decisions and policy for proper use of genetic testing information.

The next section will describe a proposal for a model for a melanoma unit using the elements and data from the study and from discussions in the document.

Proposed Model for a Multi-Disciplinary Melanoma Unit Approach

Proposing a model for a multi-disciplinary melanoma unit would not vary tremendously from the model observed in the Yale Cancer Center Melanoma Unit. The evaluative cohort study and the group of active patients both under treatment by physicians at Yale Cancer Center Melanoma Unit brought forth essential concepts

necessary to such a unit or center when treating malignant melanoma. Assessment of survival potential as is possible through imaging studies available and through diagnostic utilization methods such as in vitro tumor testing may yield very useful information to facilitate treatment regimen for the individual patient. Determining the economic constraint imposed on the individual patient together with an assessment of their issues that constitute quality of life has been shown to be important to the individual patient, while to varying magnitude and latitude for each patient.

As was discussed earlier on in this document there are examples in the literature with bacterial disease processes wherein fundamental data can be generalized regarding drug resistance and diagnostic testing and its impact of economics for the patient and population under study or treatment. The example discussed earlier of *H. pylori* emphasizes this point. According to Roman et al. (2003) the major obstacle to 100% effective eradication of *H. pylori* infection is represented by antimicrobial-resistant *H. pylori* strains. The investigators evaluated whether regimens based on pretreatment susceptibility testing (C-urea breath tests) were more effective and cost saving compared with standard triple drug therapy nonsuceptibility testing-based therapy in the eradication of *H. pylori* infection. The investigators found there were savings of $5 per patient for the patients receiving the pre-treatment antimicrobial susceptibility testing as well as 97% eradication rate seen with the testing. Mason and colleagues (1999) found DiSC assay-guided therapy may be a cost-effective use of health resources for chronic lymphocytic leukemia.

In a study conducted by Sonnenberg (1996), the investigator seeks answers as to whether all dyspeptic patients should undergo a radiologic endoscopic procedure to

diagnose peptic ulcer, or would a serologic test for *H. pylori* provide sufficient evidence to start all *H. pylori*-positive patients on empirical antimicrobial therapy? The investigator conducted a cost-benefit analysis to evaluate the questions. Successful eradication is associated with the potential benefits of healing dyspepsia and preventing peptic ulcer or gastric cancer.

In a sensitivity analysis, all of the transition rates and benefits are varied over a wide range to test the robustness of the calculated decision outcomes. The results showed that the cost-benefit relationship of serological screening for *H. pylori* in dyspeptic patients is influenced primarily by the response rate of non-ulcer dyspepsia to *H. pylori* eradication and secondarily by the monetary benefit of ulcer prevention and the prevalence rate of peptic ulcer in *H. pylori*-positive patients. A response to *H. pylori* eradication in 5-10% of all patients with non-ulcer dyspepsia would make screening and treatment for *H. pylori* a beneficial option, irrespective of any other potential benefits (Sonnenberg, 1996). According to this study, if ulcer prevention were associated with long term benefit of $4000 or more and if the ulcer prevalence rate exceeded 10% of al dyspeptic patients, serologic screening would pay off. As long as no unequivocal evidence exist that non-ulcer dyspepsia responds to eradication of *H. pylori*, treating all dyspeptic patients who test positive for *H. pylori* is not recommended according to Sonnenberg. Further, he recommends that antimicrobial therapy should be reserved to patients with proven ulcer or to patients with non-ulcer dyspepsia for whom other measures have failed.

Parsonnet et al. (1996) studied a comparison of two interventions in a cost-effective analysis to estimate the costs and benefits associate with for *H. pylori* at age 50

and treating those individuals with antimicrobials. These investigators found that screening and treatment for *H. pylori* is potentially cost effective in the prevention of gastric cancer, particularly in high-risk populations. In this case-based analysis, 11,646,000 persons in the US would be screened and 4, 658,400 treated, at cost of $996 million. Cost-effectiveness was $25,000 per year of life saved. Cost-effectiveness was sensitive to the efficacy of the cancer prevention strategy. At low efficacy rates (<10%), the screening program was more expensive or $75,000 per year of life saved. In a high-risk group such as Japanese-Americans, however, screening and treatment required less than $50,000 per year of life saved even at 5% treatment efficacy.

Several noninvasive methods are now available for diagnosing *H. pylori* infection. Because the prevalence of *H. pylori* is variable in patients requiring testing, the optimal testing strategies may vary under different conditions. One study that evaluates the cost-effectiveness of competing diagnostic strategies for *H. pylori* in patients with varying *H. pylori* prevalence was conducted by Vakil et al. (2000). The investigators discovered an interesting finding. The choice of an initial test for *H. pylori* detection in the study depended upon the prevalence of *H. pylori* infection and the value placed on increased diagnostic accuracy. Although ELISA results in the lowest cost-effectiveness ratios, in patients at low-intermediate pretest probability of infection, the stool test provides increased accuracy, with modest incremental costs.

Barbieri et al. (2005) conducted a literature search to identify economic evaluations of pharmaceuticals in two or more European countries. The studies identified were then classified by methodological type and analyzed to assess their level of variability and to identify the main cause of variation. Assessments were also made of

the extent to which differences in study results among countries were systematic and whether they would lead to a different decision, assuming a range of values of the threshold willingness-to-pay for a life-year. The conclusion shown that cost-effectiveness results for pharmaceuticals vary from country to country in Western Europe and that these variations are not systematic. In addition, constraints imposed by analysts may reduce apparent variability in the estimates. The lessons for inferring generalizability are not straightforward, although the implications of variation for decision making depend critically on the cost-effectiveness thresholds.

Human immunodeficiency virus (HIV)-infected individuals failing highly active retroviral therapy (HAART) have a substantially lower chance of clinical success than naïve patients given their first antiretroviral therapy (Lauria and Angeletti (2003). This suggests that HAART failure is a determinant for an increase in the cost of treatment. A review of the literature regarding cost and impact of antiretroviral drug-resistance testing was performed. Examination of existing methods to execute a cost-effective analysis on the use of these tests in clinical practice was also undertaken. The cost of treatment failure in HIV-infected patients had been quantified in several retrospective studies. The cost of care for patients with virological suppression was significantly lower than those with a single virology-failure. Moreover, the latter group had a lower cost than those patients with multiple failures. The result of the cost-effective analysis based on a specific model application using genotypic resistance assays to guide the choice of subsequent therapy in HIV disease, is cost-effective under a wide range of assumptions regarding effectiveness and costs. The available studies on the cost-effective evaluation of genotypic test are limited, and the respective studies supply important indications on

cost-effective evaluations (Laurie and Angelletti, 2003). Despite the demonstrated benefits, antiretroviral drug resistance testing presents failures and limitations that also restrict the cost-effective analysis.

Proposed Model and Quality of Life

Sajatovic and colleagues (2005) suggest there is emerging literature to suggest that a collaborative care model, in which patients are active managers of their illness within a supportive social environment, is a beneficial approach for individuals with bipolar disorder. The authors conducted a qualitative exploration of patient's attitudes towards the collaborative care model. They first queried individuals regarding their opinions on the ingredients for an effective patient-clinician relationship and the authors conceptualized these elements around three emerging themes: patient-centered qualities, clinician-centered qualities, and interactional qualities. Under the heading of patient-centered qualities, the individual has specific responsibilities such as coming to appointments and sharing information, whereas the clinician likewise has specific responsibilities, such as keeping abreast best practices, evidence based literature and being a good listener. Treatment adherence in this study was identified as a self-managed responsibility with the larger context of the collaborative model. While this evaluation focuses on a mental health disease, its premise can be generalized to medical illness including advanced melanoma.

Components of the psychological challenges that accompany debilitating illness especially sudden end-of-life disease are for some patients a very realistic concern that exists on a continuum during the course of treatment. Based on the evaluative study in this document both the cohort and those whose cases were presented during Yale Cancer

Center Melanoma Conference, the mental health issues facing the patient with advanced melanoma can become a large part of the medical management. It was demonstrated that quality of life issues, while not succinctly measurable for malignant melanoma, are prevalent for the patient and thus the treating physician. One cannot ignore for example, extreme depression secondary to interferon administration or lack of insurance to cover medication costs, or the sudden need to relocate housing to commence clinical trial participation across the country. The chronic illness model (Lin et al, 2005) suggests matching treatment to preference to effect outcome for patients with depression.

As a result, a model for a melanoma unit would include an approach that has built- in mechanisms to accommodate the likelihood that the patient will at some point in the short course of their disease, once diagnosed with stage III or IV melanoma confront challenges to their psychological well-being. Data collected from the cohort study revealed over one third of the patients at least on one occasion either failed to show for their appointment with the surgeon or the oncologist or discontinued their interferon or IL-2 on their own yet without informing the treating physician. This may lead to difficulties with flow of scheduling for the office and can result in difficulty for the treating physician in deciphering changes in tumor behavior and/or side effects for the patient. It would therefore be recommended to establish as a preventative measure for the patient and the treating physician, an arrangement or agreement of mutual compliance. Such a preemptive measure may occur if implemented at the outset of the diagnosis being conveyed to the patient and once a treatment plan is established.

The issue of noncompliance exists for any medical practice, yet the issues became more salient and critical for a disease with a rapid course as seen in the evaluative study.

The model that Sajatovic and colleagues evaluated for mental illness may indeed have benefit in a setting for malignant melanoma. Cited literature in this document suggests that in general, patients do prefer to become involved in their care and to be informed where possible of the details of their illness. Holding the patient accountable for compliance in the form of verbal contract may in effect provide comfort to the patient.

The treating physicians involved in the multi-disciplinary team approach have the advantage of discussing the quality of life concerns the patient may have shared with the other consultants in concentrated forum who collectively are managing the patient. This was observed in nearly every patient whose case was presented at the Yale Cancer Center Melanoma Unit conference. That the focus in many cases is devising a treatment plan around quality of life issues perhaps in lieu of extended survival that may or may not result from currently available therapies may obviate the treating physician from administering therapy without inclusion of the patients' quality of life concerns. For example, if a patient prefers palliative measure over heroic attempts to gain extended months of life in order to arrange business and family in the event of death, it is better the treating physician know this up front to establish treatment accordingly. That the individual physicians in the Yale Cancer Center Melanoma Unit group collectively took such examples into consideration, the over-all multi-disciplinary approach became one of compassion on an individual basis that over-rode in some cases, the notion of administering the strongest therapeutic regimen available.

Hansdottir and Halldorsdottir (2005) conducted a phenomenological study of elderly individuals in Iceland in an ethical and cultural framework to obtain end-of-life treatment in order to understand how they interact and influence choices of treatment. A

special emphasis was on views toward life and death as studies have indicated their importance. Results revealed end of life treatment to involve the following themes: ethical, medical, the patient's evaluation of his/her own life, the impact of the decision on loved ones and their own experiences of loss, grief and death.

The proposed model for a multi-disciplinary melanoma unit approach, as was inclusive in the Yale Cancer Center Melanoma Unit, is concurrent participation of the oncologist and surgeons and others comprising the team, in clinical trial. Somkin and colleagues (2005) examined the barriers to physician (oncologists, oncology leaders, and health plan leaders) participation in cancer clinical trials. The investigators found that the physicians who did participate expressed extremely favorable attitudes toward trials as a source of high-quality patient care and a benefit to themselves professionally. While positive attitudes towards trials were common, and were significant bivariate predictors of enrollment by the physicians evaluated, organizational factors were the predominate predictors in multivariate analysis. The best combination of factors independently predicting enrollment related to organizational support for trials, subspecialty of the oncologist, and limitations of trial eligibility requirements. The authors suggest that there is a critical need for infrastructure to support trials, especially additional support staff and research nurses. In addition, they concluded the need for better intra-organizational communication and consideration of the impact of trial design on internal health plan resources. Their research supports the need to continue an international dialogue about the broadly defined benefits and costs of clinical trials to patients, physicians, and health plans. Having a medical team with active participation and acute awareness as to state of the art clinical trial for the malignancy they are treating is optimal for the patient.

Keeping abreast of international treatment in the U.S. and abroad through international conference attendance demonstrates expressed interest in shared education in evidentiary medicine.

The proposed model would include recruitment of clinicians into the multi-disciplinary group who have experience with integrative medicine. As was true of the evaluative cohort study, patients who face end-of-life decisions regarding treatment options may choose alternative (non allopathic) medicine such as the patient who elected to take medication from the Dominican Republic. The treating physicians in the group continued to provide surgical treatment to the patient and arranged communication with the physician in the Dominican Republic to learn dosing schedule for the alternative medication. In this way, the team remained open to choices of treatment as dictated by the patient's wishes yet did provide a detailed explanation of the suggested immunobiotherapy for the patient's stage III melanoma.

Earlier in this document, it is stated that in order to initiate treatment for patients diagnosed with malignant melanoma, it is recommended that all patients be entered into clinical trials. In the proposed model, it is suggested that the experts on the multi-disciplinary team also participate in clinical trial as a means to stay current with trail mechanism and the challenges presently facing patient participation. The surgeon and oncologist were involved in the Sunbelt Oncology Trial at the time of the evaluative study. The observations made in conference and information from the interviews with these physicians demonstrated additional commitment to patient care and continued professional education. The nurses employed by the physicians in the group had

additional training to administer medical regimens and could offer explanation as to administrative needs in behalf of the patients who receive medication on site.

Logistic Model for Binary Short-Term Outcomes to Study Melanoma Unit

A future data analysis where the goal is to estimate the efficacy of treatment for malignant melanoma and subsequent benefit of a multi-disciplinary clinical approach to such treatment may be performed using a logistic model for binary short-term outcomes. In the analysis, data may consist of binary outcomes (patient satisfaction/no satisfaction) on each of a group of patients randomized to treatment (low and high dose) or placebo (if feasible). Data characteristics could indicate that the 1) treatment effects vary non-linearly with time; 2) there is substantial heterogeneity across subjects in their responses to treatment, and 3) there is a high proportion of subjects who never experience any relief (the non-responders) regardless of treatment chosen, whether based solely on quality of life issues particular to the patient or strictly on attempting to prolong survival via administration of biochemotherapy or like modality.

To overcome these challenges, a hierarchical model for binary short-term data with a mixture distribution on the probability of response to account for the high frequency of non-responders that may be anticipated based on the evaluative study conducted on both the cohort and patient cases presented in the Yale Cancer Center Melanoma Unit evaluation. While such a model would be specified conditionally on subject-specific variables, inferences on key population-average parameters for the assessment of the treatment's efficacy in a population could be formulated. In addition, a model-checking method to compare the goodness-of-fit of the model against other

clinical approaches for aggregated counts, such as the zero-inflated Poisson and zero-inflated negative binomial models may serve as evaluative measures.

Choi et al. (2005) found that treatment is effective with respect to placebo with higher efficacy at the beginning of the study of chronic gastrointestinal disturbance. However, the investigators found that models for aggregated counts cannot capture time trend such as the initial treatment benefit versus the development of tolerance during the early stage of treatment which may be important information to physicians to predict the treatment effects for their patients. In patients treated with malignant melanoma a similar difficulty in obtaining measure of efficacy become apparent, not because of tolerance but as result of toxic or unwanted side effects that alter a patient's desire to continue treatment or medical care for their terminal disease.

Risk adjustment techniques may be employed to account for the health status of patients when predicting or explaining the costs of health care for defined populations or for evaluating retrospectively the performance of providers who care for them. Such techniques may be useful for future studies evaluating a model for the multi-disciplinary clinical approach to malignant melanoma (Blumenthal, et al. 2005). Although the federal government in the US seems to have settled o an approach to risk adjustment for Medicare Advantage programs, adoption and implementation of risk adjustment techniques elsewhere in US health care markets have proceeded much more slowly than was anticipated. Blumenthal et al. evaluated case studies in six markets (Baltimore, Seattle, Denver, Cleveland, Phoenix, and Atlanta) as of 2001. The results found that for health policy in general, the differing experiences of public and private health care

sectors with risk adjustments serve as markers of the divergent paths that public and private health care sectors are pursuing with respect to managed care and risk sharing.

Conclusions to Chapter 5

Predominate in the development of a model for a melanoma unit for patients with malignant melanoma is the accommodation of the interpersonal manner of the treating physicians who are devising the treatment plans. Malignant melanoma currently has no definite standard treatment regimen shown to establish cure of even improve survival. Until such time where improvement is made, attention to the dynamic between the treating physician who is skilled in a heuristic clinical approach and utilizes evidentiary medicine and diagnostic tools in concert with economics and quality of life issues for the individual patient offers optimal focus and will best serve the patient.

Tat and Barr (2005) compared patient's satisfaction with outpatient care in a traditional clinic and a western-style clinic in Ho Chi Mihn City, Vietnam. As Vietnam opens its economy to privatization, its system of healthcare will face a series of crucial tests (Tat, 2005). Vietnam's system of private healthcare-once comprised of individual physicians holding clinic hours in their homes-has come to also include larger customer-oriented clinics based on an American business model. As the two models compete in the expanding private market, it becomes increasingly important to understand patients' perceptions of the alternate models of care. The study by Tat and Barr reported on interviews with 194 patients in two different types of private-sector clinics in Vietnam: a western-style clinic and a traditional style, after-hours clinic. In bivariate and multivariate analysis, the investigators found that patients at the western style clinic reported both higher expectations of the facility and higher satisfaction with many aspects

of care than patients at the after-hours clinic. These different perceptions appeared to be based on the interpersonal manner of the physician seen and the clinic's delivery methods rather than the perceptions of the physician's technical skill and method of treatment. According to the investigators, these findings were unaffected by the ethnicity of the physician seen. These findings suggested to the investigators that patients in Vietnam recognize and prefer more-customer oriented care and amenities, regardless if physician ethnicity and perceived no significant differences in technical skill between the private delivery models.

While the study does offer what seems to be a human tendency towards a patients desire for satisfying interpersonal relations with their treating physician, and this desire seems universal, there is concurrent to this mannerism a demand for the treating physician to maintain skills to practice medicine with an astute ability to discern utilization of diagnostic tools, the research that evaluates their efficacy, accuracy, feasibility and their economic benefit. Quality of health care depends, among others, on the quality of a physician's domain of knowledge (Braum et al. (2005). To handle exceptional cases, such as treating advanced stage III and IV malignant melanoma, assess to the full range of medical literature is required and well as a fund of knowledge with evidence-based studies.

Clinical guidelines for improving the quality of life and the quality of care are a familiar aspect of clinical practice. Formal consensus models such as the nominal group technique used in psychodynamic interpersonal therapy (Raine et al., 2005) are often used as part of guideline development, but little is known about factors that influence the statements produced by the nominal groups, and on their consistency within the research

evidence. There are few studies in the medical literature which review or evaluate a multi-disciplinary approach to clinical treatment. One study (Udenzue et al., 2005) identifies the increasing incidence of diabetes mellitus worldwide making traditional approaches to its management inadequate according to the investigators. The involvement of young people in this diabetic "epidemic" provides an opportunity to apply a multidisciplinary approach to its management, to help reduce the large burden of the disease and its complications. In 1998, Udezue and colleagues established a diabetic clinic for young adults in Saudi Arabia, located within a privately owned company health center, because according the investigators the patients were not receiving adequate attention in the adult clinic. The purpose was to optimize diabetes control by teaching about diet, exercise, medications, and other practical diabetic management issues. Diabetic control as measured by serial glycosylated hemoglobin levels (HbA1c) and the occurrence and severity of diabetic ketoacidosis improved in the patients studied (n=105) over the first four years of clinic operation. Diabetic control improved over those four years in the patients. Studies in the West have shown that small reductions in HBA1c have translated into dramatic decreases in microvascular complications. These results offered the investigators to suggest application of the model to a larger population group to learn whether further study may help determine whether to adopt this pattern of care more widely, with its suggested benefits in the reduction of diabetic morbidity, mortality and health care cost.

A common problem with clinical research on diagnostic testing of in vitro tools including biomarker assays is in determining the optimal frequency of testing to minimize cost (Judd et al, 2003). The financial and health benefits of reducing the time

from diagnosis to potential determination of drug resistance in testing such as EDR in vitro studies are ideal parameters to evaluate for future studies.

Guidelines for treatment in a disease that at present offers little in the way of cure may be best developed using the expertise of a multi-disciplinary heuristic clinical approach based on evidentiary medicine and clinical experience. Such guidelines cannot be based on evidentiary data alone as clinical judgment which offers transparent and structured nuance are needed to support patient preference and the needs and realities of the disease process as experienced by the individual patient.

The global problem of antimicrobial resistance and anti-tumor resistance is pressing in developed countries and particularly developing countries where infectious disease burden is high and cost constraints prevent the widespread application of newer, more expensive agents and novel diagnostic testing. Gastrointestinal, respiratory, sexually transmitted, and nosocomial infections are the leading causes of death in the developing world, and management of all these conditions has been critically compromised by the appearance and rapid spread of resistance (Okeke, 2005). Surveillance rates of resistance in many countries including the U.S is suboptimal, the general picture though is one of accelerating rates of resistance.

Melanoma is the most aggressive form of skin cancer and is notoriously resistant to all current modalities of cancer therapy (Soengas and Lowe, 2003). According to Soengas and Lowe (2003), "A large set of genetic, functional and biochemical studies suggest that melanoma cells become 'bullet-proof' against a variety of chemotherapeutic drugs by exploiting their intrinsic resistance to apoptosis and by reprogramming their proliferation and survival pathways during melanoma progression." The challenge now is

to devise strategies potent enough to compensate or bypass theses cell death effects and improve the actual poor prognosis of patients at late stages of the disease.

Similar to the aforementioned infections, malignancies also show extreme drug resistance leading to morbidity and mortality. Considerable economic burden and health care concerns arise from difficulties in treating bacterial and tumor cell resistance. Future research should focus on identifying economically viable diagnostic tools that accurately predict extreme drug resistance in malignant melanoma and augment the efficacy of treatment. However until further progress is realized, the emphasis must focus on a treatment approach that integrates individual patient needs with the expertise of the treating physician. Treatment for advanced melanoma at present is derived from an integration of clinical experience, evidentiary medicine and physician intuition.

As novel diagnostic tools continue to enter the clinical realm and are identified as reliable predictors of advanced stage III and IV melanoma, so continues the unified goal amongst patients, researchers and physicians who treat the disease. With rapid improvements in drug design, there is optimism for the development of better therapeutic options for patients with malignant melanoma. It is through continued collective efforts that this goal be realized.